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PRINCIPAL INVESTIGATOR: Hye-Sook Seo, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77030

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Abstract

Retinoids are known by their ability to inhibit tumor growth and metastasis in breast cancer indicating their promising chemo-preventive and therapeutic activity. Retinoids exert their biological functions through two receptors, RAR and RXR. RXR-bound compounds (rexinoid) suppress ER-positive and ER-negative mammary tumors with reduced toxicity compare to RAR-bound compounds. Among RXR receptor isoforms, RXR alpha seems to play a critical role on tumor suppresion. Thus, we focussed on alpha using RXR alpha specific ligand, Ro25-7328, to study whether this rexinoid suppresses breast cell growth. We found that Ro25-7328 suppressed the growth of both normal HMEC and T47D breast cancer cells.

To identify genes that are regulated by RXR alpha, we treated HMEC with Ro25-7328, then examined changes in gene expression using affymetrix microarray. In HMEC, we identified 638 genes up-regualted and 347 genes down-regulated by Ro25-7328 with changes in fold induction (more than 2 fold). Among them, we found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus; i.e., integrin beta4, integrin alpha6, BAX, E-cadherin, FOXO3A, paxillin, STAT3, CDC42. Further focussing on the implication of those genes in Ro25-7328-induced growth suppression, we would clarify the mecahnism by which rexinoid suppresses breast cancer development. – **This work is presented by poster in 2005 Era of Hope meeting, June 8 – June 11, 2005 (poster number # 36-13)**

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INTRODUCTION

Breast cancer is the most diagnosed cancer in women and the second leading cause of death from cancer in women (Jemal et al., 2005). In 2005, the ACS estimates 211,240 women will be newly diagnosed with breast cancer, and 40,410 women will be died from this disease (Jemal et al., 2005). Ultimate purpose of scientist and clinicians to conquer this disease is to prevent breast cancer incidence, early detect and treat breast cancer with effective and appropriate therapy resulting in long overall survival minimizing side effects. The purpose of my project is to find the way to prevent and treat breast cancer using retinoids. Retinoids play an important role in inhibition of tumor growth and metastasis in breast cancer indicating their promising chemo-preventive and therapeutic property. Especially, RXR-bound retinoid (rexinoid) suppress ER-positive and ER-negative mammary tumors with reduced toxicity compared to RAR-bound compounds (Gottardis et al., 1996; Wu et al., 2002a, 2002b). Rexinoids are also active in animals in tamoxifen-resistant breast cancer (Bischoff et al., 1999; Lippman and Lotan, 2000) and in all-trans-RA-resistant breast cancer cells (Crowe and Chandraratna, 2004). These suggest that it is worthy to work on rexinoids to test their ability to prevent normal breast cell growth and breast cancer cell growth, and further clarify the mechanism by which rexinoids suppress breast cancer development. For that purpose, we identified genes regulated by rexinoid, and selected several target genes that could be involved in rexinoid-induced growth inhibition. From this finding, we may discover by which pathway rexinoid exerts its tumor-suppressive activity, and we may find the way to prevent and treat breast cancer.

BODY

Retinoid receptors are expressed in normal and malignant breast epithelial cells and are critical for normal development (Decensi et al., 2003). Retinoid receptors are ligand-dependent transcription factors which modulate gene expression upon DNA binding. The two families of retinoid receptors (RARs and RXRs) exist with three isotypes, α, β, γ, encoded by separate genes, and represents numerous alternatively spliced variants. The retinoids are active as dimers (heterodimers or homodimers); RARs heterodimerize with RXRs, RXRs heterodimerize with multiple members of steroid receptors family, such as RAR, thyroid hormone receptor (TR), Vitamin D receptor (VDR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR) and pregnane X receptor (PXR) suggesting its involvement in several signaling pathways. RXR also homodimerize in transfected cells (Zusi et al., 2002).

For the first year of the grant research, we generally followed the approved statement of work using the noticed breast cell lines, but using different RXR-bound compound. We previously focused on LGD1069 and LG100268 compounds (rexinoids) which randomly bind to RXR isoforms and planed to work on LGD1069-target gene IGFBP-6 to investigate the role of this gene in breast cancer suppression induced by LGD1069 (statement of work). But in the first period of this study, we used another specific rexinoid, Ro25-7328 which exclusively bind RXR α isoform. Because, RXR α seems to play a critical role in tumor suppression. It was reported that over-expression of RXR α sensitized breast cancer cell lines to the anti-proliferative effect of RXR-selective ligand (Crowe and Chandraratna, 2004), moreover, infection of adenoviral RXR α induced nucleoplasmic over-expression of RXR α and resulted in apoptosis under treatment with an RXR ligand in retinoid-resistant MDA-MB-231 (Tanaka et al., 2004). Hence, we focused on RXR α isoform to see its peculiar role in tumor suppression identifying genes regulated by Ro25-7328. Human RXR α gene spans over 40 kilobases in size and consists of at least 10 exons separated by introns ranging in size from 700 base pairs (intron 3) to more than 7.8 kb (intron 4) (Li et al., 2000).

Result 1. Expression of RXR α in breast cell lines

We first determined the expression of RXR α in 1 normal breast cells (human mammary epithelial cells – HMEC) and 2 retinoid-sensitive breast cancer cells (MCF-7 and T47D) and 2 retinoid-insensitive breast cancer cells (MDA-MB-231 and MDA-MB-435). We found that all breast cell lines express RXR α but with different intensity. MCF-7 and T47D expressed higher amount of RXR α - (Figure 1 - Figure 2 in poster).

Interestingly, ER-negative breast cancer cells which do not respond to retinoid treatment such as MDA-MB-231 and MDA-MB-435 also expressed RXR α. **This suggests that RXR α is non-functional losing DNA binding activity or failing to recruit essential co-activators required for the gene activation. Different and inappropriate sub-**

localization of the receptor may also explain the unresponsiveness of the cells to retinoid.

Secondly, we tested the anti-proliferative effect of RXR α specific compound, Ro25-7328 in HMEC cells and retinoid-sensitive cells, MCF-7 and T47D. By MTT assay, we found that Ro25-7328 suppressed HMEC normal breast cell growth by dose-dependent manner. (Figure 2 - see Figure 3 in poster). We also found that Ro25-7328 suppressed T47D cell growth at 1 μM (Figure 3 - see Figure 4 in poster). Ro25-7328 induced mild suppression of the cell growth in MCF-7 at 1 μM although MCF-7 cells expressed the highest quantity of RXR α (Figure 4). Besides, all-*trans* RA, 9-*cis*-RA and 13-*cis*-RA have the tendency to inhibit cell growth in HMEC, and strongly inhibit the cell growth in T47D. **These results indicate that RXR α agonist, Ro25-7328 suppressed the growth of HMEC and retinoid-sensitive breast cancer cells, T47D and MCF-7. Hence, RXR α seems to play an important role "at least in part" for growth suppression induced by rexinoid in breast cells. Moreover, the amount of RXR α receptor may not be related to the inhibition of breast cell growth induced by Ro25-7328.**

Next plan of the related study;
*We did not yet perform MTT assay on retinoid-insensitive breast cancer cell lines, MDA-MB-231 and MDA-MB-435, although we suppose that RXR α–specific ligand, Ro25-7328 would not influence the cell growth in both cell lines. This work will be completed in the next term and we may further investigate the function of RXR α in retinoid-*insensitive cell lines.

Result 2. Determine RXR α target genes of HMEC by Affymetrix microarray

For the next step of the study, we identified genes regulated by RXR α–specific ligand, Ro25-7328 in normal breast cells. Gene expression profiles were established by using Affymetrix microarray (human genome U133A 2.0). For that purpose, we treated HMEC cells with Ro25-7328 using the concentration which most strongly suppressed the cell growth (10 μM), and total RNA sample was harvested after 12h; this time point was selected for study since retinoid treatment would likely regulate the expression of genes earlier than 24h (Ma et al., 2003). We then examined changes in gene expression by using the microarray to investigate which genes are related to cell growth inhibition induced by the RXR α agonist. **In HMEC, we identified 638 genes up-regulated and 347 genes down-regulated by Ro25-7328 with changes in fold induction greater than 2 fold.** 22 genes were strongly up-regulated (more than 10 fold), and 5 genes were strongly down-regulated (more than 4 fold) in expression levels by Ro25-7328 (Table 1 and 2 - see Table 1 and 2 in poster).

Among them, we found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus; i.e., integrin beta 4, integrin alpha 6, cadherin 1, type 1, E-cadherin (epithelial) (CDH1), paxillin

(PAX), BCL2-associated X protein (BAX), forkhead box O3A (FOXO3A), signal transducer and activator of transcription 3 (STAT3), collagen, type VI, alpha 3, cell division cycle 42 (CDC42), etc. We selected our major genes of interest by referring to PathArt program which demonstrate the relationship between genes by several signaling pathways (Figure 5-8 - see Figure 5-8 in poster).

Result 3. Confirm the changes of modulation of RXR α target genes of HMEC by real-time RT-PCR and western blot analysis

The induction of a total of 7 genes by rexinoid was confirmed by real-time RT-PCR assays including integrin beta 4, integrin alpha 6, E-cadherin (CDH1), PAX, BAX, FOXO3A, STAT3; up-regulation of those genes by Ro25-7328 was confirmed (Figure 9 - see Figure 9 in poster). The changes in fold induction of integrin beta 4, integrin alpha 6, E-cadherin (CDH1), PAX, BAX, FOXO3A, STAT3were confirmed by western blot analysis; up-regulation of BAX, E-cadherin, interleukin alpha 6 and the down-regulation of CDC42 were confirmed (Figure 10 - see Figure 10 in poster).

Next plan of the related study;
We need to confirm some more down-regulated genes by real-time RT-PCR and western blot analysis – i.e., collagen, type VI, alpha 3. This confirmation will be completed in the next term.

Among selecting genes, we are especially interested in CDH1, FOXO3A and BAX.

BAX belongs to bcl-2 family which is involved in apoptotic pathway. Among the members of this family, Bcl-2 and Bcl-XL act as anti-apoptotic regulators, while Bax and Bak act as pro-apoptotic regulators. Over-expressed Bax is transported to the mitochondria where it induces the release of cytochrome c. Released cytochrome c binds Apaf-1 and caspase-9, and induces apoptosis in cells (Finucane et al., 1999; Ikeguchi et al., 2002). On the other hand, Bcl-2 binds to Bax and can form heterodimers, thus inhibiting Bax activity. The ratio of Bcl-2 to Bax protein has been reported to be correlated with apoptosis in cancer cells (Ikeguchi et al., 2002; Oltvai et al., 1993). Reduced levels of Bax proteins have been associated with poor responses to chemotherapy and shorter overall survival in women with metastatic breast cancer (Redondo et al., 2003; Krajewski et al., 1995).

E-cadherin is a cell-to-cell adhesion glycoprotein and potent invasion/tumor suppressor. This protein consists of large extracellular domain comprising five cadherin-motif subdomains, a single-pass transmembrane segment and a short conserved cytoplasmic domain, which interacts with several proteins, termed catenins (Berx and Van Roy, 2001). E-cadherin is expressed in normal breast epithelium, benign breast lesions and breast carcinoma (Harigopal et al., 2005). The loss of E-cadherin expression or function in epithelial carcinomas seems to disrupt tight epithelial cell-cell contacts resulting in release of invasive tumor cells from the primary tumor. Hence, E-cadherin is believed to

serve as a widely acting suppressor of invasion and growth of epithelial cancers (Hazan et al., 2004). Human E-cadherin gene (CDH1) contains 16 exons, bridges a region of 100 kbp and was localized by different approaches on chromosome 16q22.1 (Mansouri et al., 1988; Natt et al., 1989; Berx et al., 1995; Berx et al., 1998). Loss or mutations of CDH1 are involved in cancers for breast, ovary, endometrium, stomach and thyroid (Graff et al., 1995; Berx et al.,1998; Berx and Van Roy, 2001).

Mammalian forkhead members of the class O (FOXO) transcription factors, including FOXO1, FOXO3a, FOXO4 are implicated in the regulation of a variety of cellular processes, including the cell cycle, apoptosis, DNA repair, stress resistance, and metabolism. FOXO proteins are negatively regulated by the phosphatidylinositol 3-kinase-Akt signaling pathway, which is activated by growth factors and cytokines. Similar to the tumor suppressor p53, FOXO is activated by stress, and induces the expression of genes that contributes to cell-cycle arrest, suggesting that it also functions as a tumor suppressor (Furukawa-Hibi et al., 2005).

Result 4. Determine RXR α target genes of MCF-7 by Affymetrix microarray

We also identified genes regulated by RXR α in MCF-7, retinoid-sensitive cell line. RXR α compound, Ro25-7328 induced mild growth suppression in this cell line. Gene expression profiles were established by using Affymetrix microarray (human genome U133A 2.0). We treated MCF-7 cells with Ro25-7328 using the same concentration used for HMEC (10 μM), and total RNA sample was harvested after 12h. We then examined changes in gene expression by using the microarray to investigate which genes are related to cell growth inhibition induced by the RXR α agonist. **In MCF-7, we identified 83 genes up-regulated and 98 genes down-regulated by Ro25-7328 with changes in fold induction greater than 2 fold** (Table 3 and 4).

Among them, we found several interesting genes which could be involved in cell growth inhibition induced RXR α agonist, Ro25-7328. i.e., transforming growth factor, beta 2, protein kinase C, delta binding protein, cathepsin S, transforming growth factor, beta 1 (Camurati-Engelmann disease), basigin, myeloid cell leukemia sequence 1 (BCL2-related) (MCL-1), BCL2-like 1(BCL2L1). We selected our major genes of interest by referring to PathArt program which demonstrate the relationship between genes by several signaling pathways (Figure 11-12). We found little overlapping genes induced by RXR α agonist between HMEC and MCF-7.

Next plan of the related study;
We need to confirm the regulation of selected RXR α -target genes by real-time RT-PCR and western blot. This confirmation will be completed in the next term.

KEY RESEARCH ACCOMPLISHMENTS

1. Find the growth suppressive activity of RAR a agonist, in HMEC, T47D and MCF-7.
2. Gene profiling for RXR α-target genes in normal HMEC.
3. Gene profiling for RXR α-target genes in MCF-7.

REPORTABLE OUTCOMES

Some part of this work is presented in **2005 Era of Hope meeting (poster presentation- #poster number; p36-13 #abstract – proceedings ; pp 253)**

CONCLUSION

To reach our ultimate goal – study the molecular mechanism by which retinoids suppress breast cancer development, we focused our attention on RXR-specific ligands (rexinoids) which have been reported to suppress breast cancer development with minimal toxicity compare to RAR-specific ligands. We also oriented our study toward RXR α isoform, which plays an important role in tumor suppression.

We found that RXR α agonist Ro25-7328 suppressed the growth of breast cells - normal HMEC cells and retinoid-sensitive breast cancer cell lines (MCF-7 and T47D). Gene profiling using Affymetrix microarray, we identified several interesting RXR α-target genes in both HMEC and MCF-7.

In HMEC, we identified 638 genes up-regulated and 347 genes down-regulated by Ro25-7328 with changes in fold induction greater than 2 fold. We selected several interesting genes, and confirmed their regulation levels by real-time RT-PCR and/or western blot analysis – they include integrin beta 4, integrin alpha 6, E-cadherin (CDH1), PAX, BAX, FOXO3A, STAT3 and CDC42.

In MCF-7, we identified 83 genes up-regulated and 98 genes down-regulated by Ro25-7328 with changes in fold induction greater than 2 fold. We selected several interesting genes, and need to confirm their regulation levels by real-time RT-PCR and/or western blot. Our selecting genes include transforming growth factor, beta 2, protein kinase C, delta binding protein, cathepsin S, transforming growth factor, beta 1 (Camurati-Engelmann disease), basigin, MCL-1, BCL2L1.

All those selected might to be implicated in growth inhibition of breast cells induced by Ro25-7328. Profound investigation of our interesting genes - CDH1, FOXO3A and BAX (HMEC) and cathepsin S, TGFβ2, basigin, MCL-1 and BCL2L1 (MCF-7), may lead us to clarify how RXR α agonist functions to inhibit breast cell growth.

This study could help us to find new preventive/therapeutic target for breast cancer, and may contribute to develop novel molecule which could inhibit breast cancer development.

We will secondly test the effect of other RXR α agonists – LGD1069 and LG100268 on breast cell growth as we mentioned in our statement of the work. They are well-known rexinoids which inhibit tumor development, and we will study their ability to inhibit breast cancer development in our system.

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Expression of RXR alpha in breast cells (western blot analysis) - normal (HMEC) and breast cancer cells

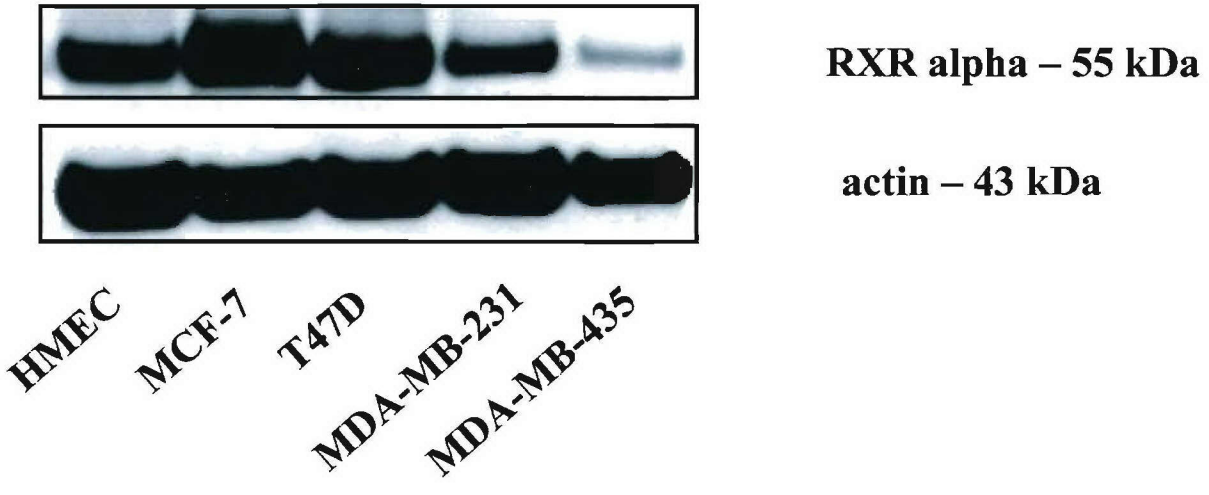


Figure 1

Effect of RXR alpha agonist Ro25-7325 on HMEC normal breast cell growth (MTT assay)

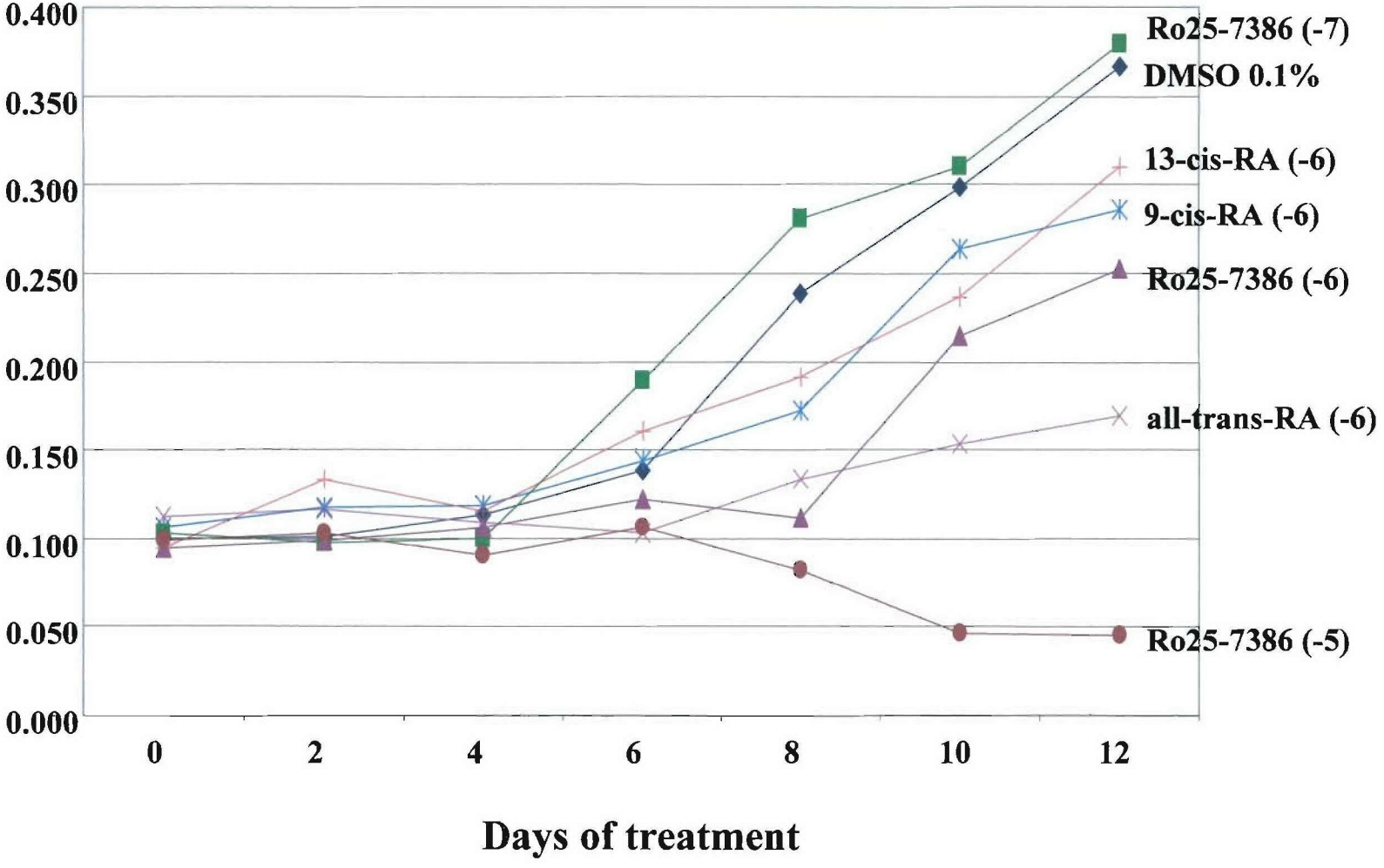


Figure 2

Effect of RXR alpha agonist Ro25-7325 on T47D breast cancer cell growth (MTT assay)

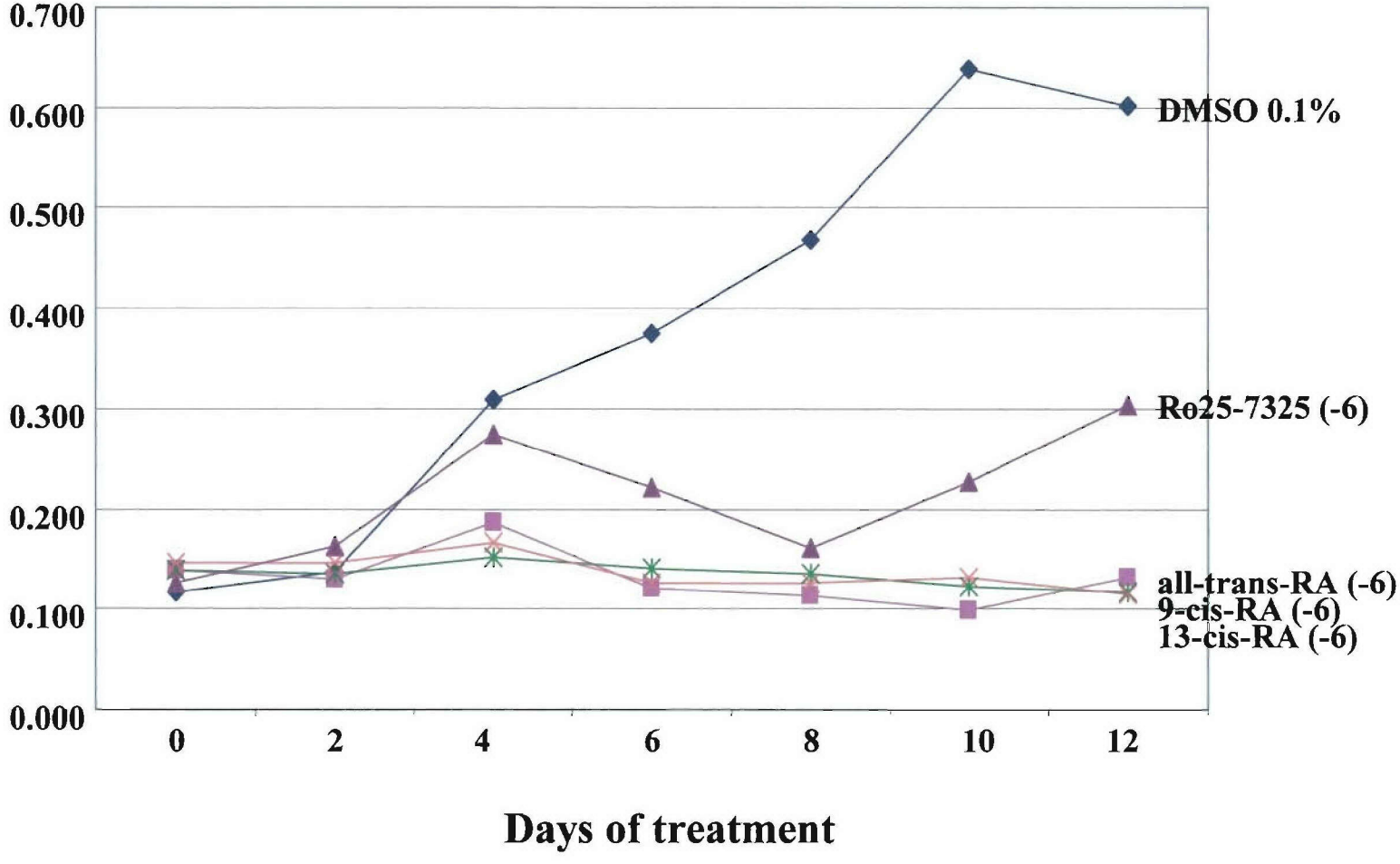


Figure 3

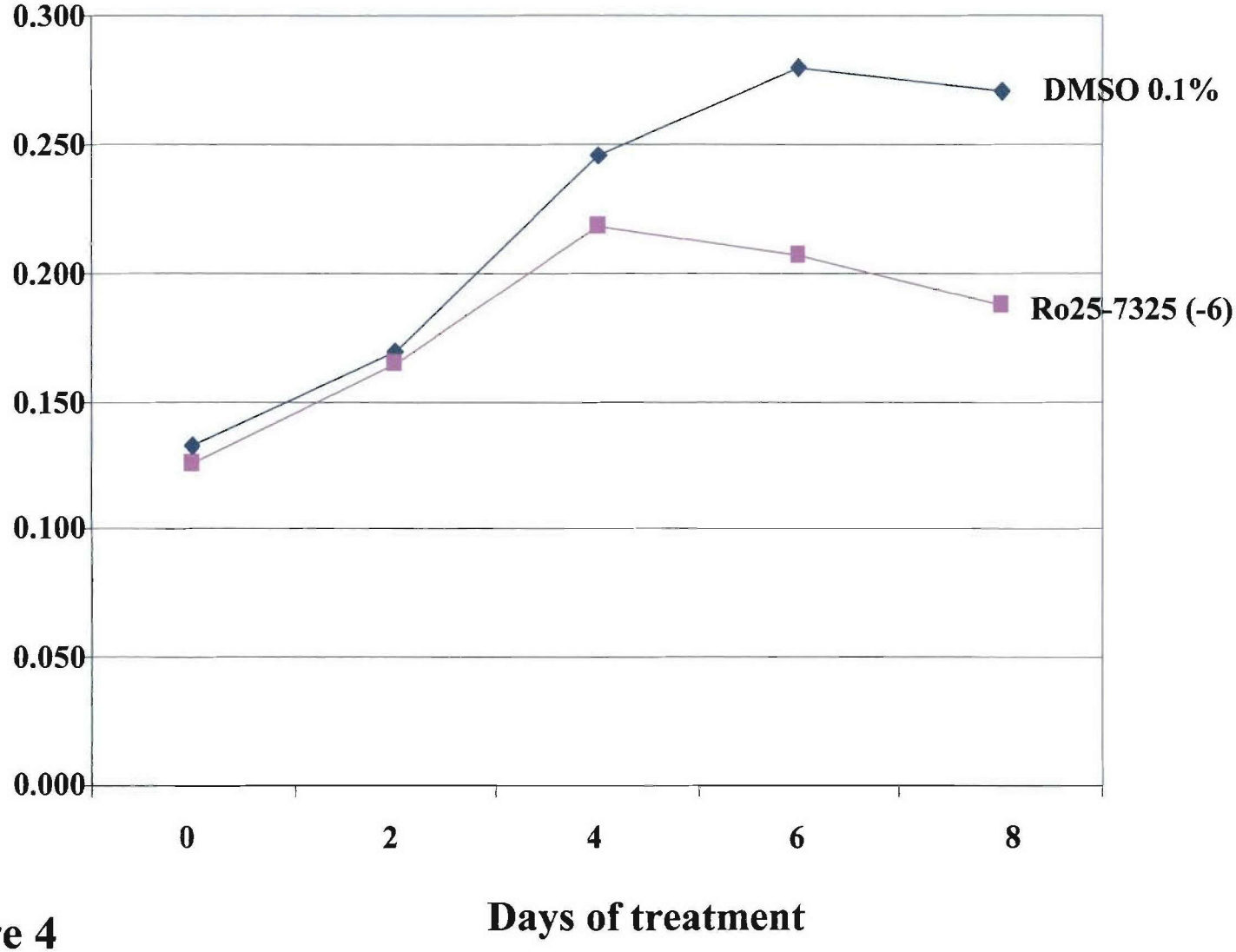

Effect of RXR alpha agonist Ro25-7325 on MCF-7 breast cancer cell growth (MTT assay)
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
2
4
6
8
DMSO 0.1%
Ro25-7325 (-6)
Days of treatment

Figure 4

Table 1

Genes up-regulated by Ro25-7386 in HMEC

Probe Set	Gene Name	Fold Change	Gene Functions
204989_s_at	**integrin, beta 4**	**26.6**	**cell-matrix adhesion, integrin-mediated signaling pathway**
201130_s_at	**cadherin 1, type 1, E-cadherin (epithelial) (CDH1)**	**20.66**	**cell-to-cell adhesion protein, potent invasion/tumor suppressor**
203392_s_at	C-terminal binding protein 1	16.35	L-serine biosynthesis, negative regulation of cell proliferation
216971_s_at	plectin 1, intermediate filament binding protein 500kDa	15.17	cytoskeletal anchoring
215780_s_at	SET translocation (myeloid leukemia-associated)	12.51	DNA replication, negative regulation of histone acetylation
201971_s_at	ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A	11.75	ATP synthesis coupled proton transport, proton transport
204426_at	coated vesicle membrane protein	11.74	intracellular protein transport
215177_s_at	**integrin, alpha 6**	**10.97**	**cell-matrix adhesion, cell-substrate junction assembly**
211905_s_at	**integrin, beta 4**	**10.34**	**cell-matrix adhesion, integrin-mediated signaling pathway**
201048_x_at	RAB6A, member RAS oncogene family	10.03	protein transport, small GTPase mediated signal transduction
214701_s_at	fibronectin 1	10.01	acute-phase response, cell adhesion, cell migration, metabolism
202118_s_at	copine III	9.48	lipid metabolism, vesicle-mediated transport
217234_s_at	villin 2 (ezrin)	9.09	cellular morphogenesis, cytoskeletal anchoring
202855_s_at	solute carrier family 16 (monocarboxylic acid transporters), member 3	9.06	monocarboxylic acid transport, transport
210935_s_at	WD repeat domain 1	8.44	perception of sound
208750_s_at	ADP-ribosylation factor 1	7.31	intracellular protein transport
202856_s_at	solute carrier family 16 (monocarboxylic acid transporters), member 3	7.26	monocarboxylic acid transport, transport
200796_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	7.25	cell differentiation, regulation of apoptosis
213606_s_at	Rho GDP dissociation inhibitor (GDI) alpha	7.25	Rho protein signal transduction, negative regulation of cell adhesion
201373_at	plectin 1, intermediate filament binding protein 500kDa	7.19	cytoskeletal anchoring
208057_s_at	GLI-Kruppel family member GLI2	7.04	organogenesis, regulation of transcription-DNA-dependent
217294_s_at	enolase 1, (alpha)	6.99	glycolysis, negative regulation of cell growth
200806_s_at	heat shock 60kDa protein 1 (chaperonin)	6.69	mitochondrial matrix protein import, protein folding
211823_s_at	**paxillin (PAX)**	**5.75**	**cell motility, cell-matrix adhesion, signal complex formation**
211833_s_at	**BCL2-associated X protein (BAX)**	**3.65**	**apoptosis, negative regulation of cell cycle**
210655_s_at	**forkhead box O3A (FOXO3)**	**3.33**	**apoptosis, cell growth and/or maintenance**
208992_s_at	**signal transducer and activator of transcription 3 (STAT3)**	**2.62**	**cell motility, intracellular signaling cascade, neurogenesis**

Table 2

Genes down-regulated by Ro25-7386 in HMEC

Probe Set	Gene Name	Fold Change	Gene Functions
201438_at	**collagen, type VI, alpha 3**	**-4.59**	**cell adhesion, muscle development, phosphate transport**
204634_at	NIMA (never in mitosis gene a)-related kinase 4	-3.59	mitosis, protein amino acid phosphorylation
209788_s_at	type 1 tumor necrosis factor receptor shedding aminopeptidase regulator	-3.48	adipocyte differentiation, antigen processing
205802_at	transient receptor potential cation channel, subfamily C, member 1	-3.43	calcium ion transport, cation transport
202149_at	neural precursor cell expressed, developmentally down-regulated 9	-3.39	actin filament bundle formation, regulation of cell cycle
204415_at	interferon, alpha-inducible protein (clone IFI-6-16)	-3.29	immune response, response to pest, pathogen or parasite
210017_at	mucosa associated lymphoid tissue lymphoma translocation gene 1	-3.12	activation of NF-kappaB-inducing kinase, anti-apoptosis
205420_at	peroxisomal biogenesis factor 7	-3.05	protein transport
219317_at	polymerase (DNA directed) iota	-3.01	DNA repair
204176_at	kelch-like ECT2 interacting protein	-3	cytoskeleton organization and biogenesis
203741_s_at	adenylate cyclase 7	-2.95	cAMP biosynthesis, intracellular signaling cascade
204078_at	synaptonemal complex protein SC65	-2.9	synaptonemal complex formation
203881_s_at	dystrophin (muscular dystrophy, Duchenne and Becker types)	-2.88	cytoskeletal anchoring, muscle contraction
209717_at	ecotropic viral integration site 5	-2.87	cell proliferation, development
213473_at	BRCA1 associated protein	-2.86	negative regulation of signal transduction
215949_x_at	immunoglobulin heavy constant mu	-2.83	immune response
205668_at	lymphocyte antigen 75	-2.83	endocytosis, immune response, inflammatory response
219688_at	Bardet-Biedl syndrome 7	-2.82	visual perception
218094_s_at	chromosome 20 open reading frame 35	-2.82	protein transport
207845_s_at	anaphase promoting complex subunit 10	-2.8	cell cycle, mitosis, ubiquitin cycle
202265_at	B lymphoma Mo-MLV insertion region (mouse)	-2.79	cell growth and/or maintenance, chromatin modification
208920_at	sorcin	-2.79	heart development, intracellular iron ion storage
218901_at	phospholipid scramblase 4	-2.78	blood coagulation, phospholipid scrambling
208883_at	E3 identified by differential display	-2.75	cell proliferation, ubiquitin cycle
209717_at	ecotropic viral integration site 5	-2.87	cell proliferation, development
218002_s_at	chemokine (C-X-C motif) ligand 14	-2.53	cell-cell signaling, chemotaxis, immune response
208727_s_at	**cell division cycle 42 (GTP binding protein, 25kDa)**	**-2.25**	**protein transport**

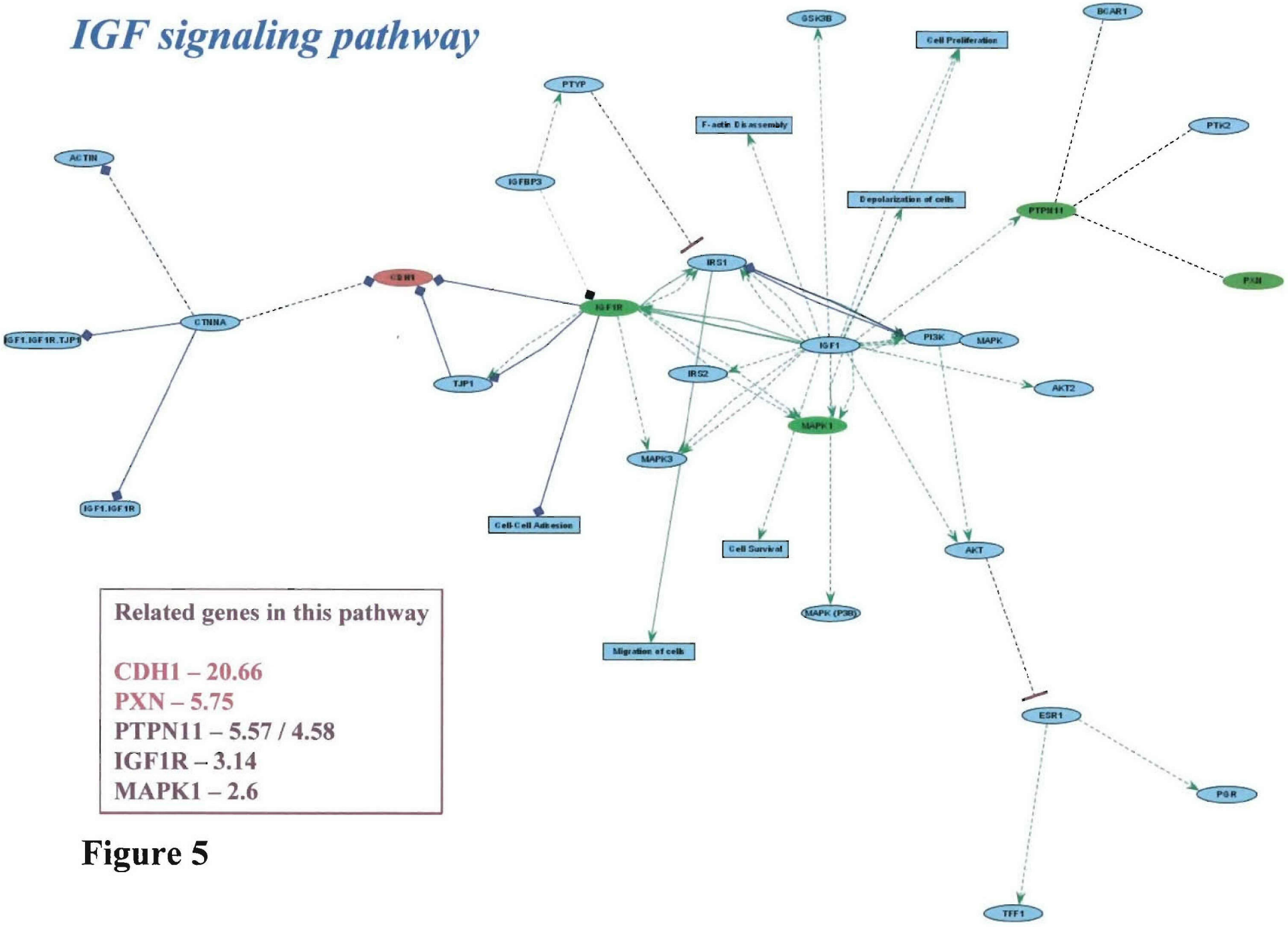
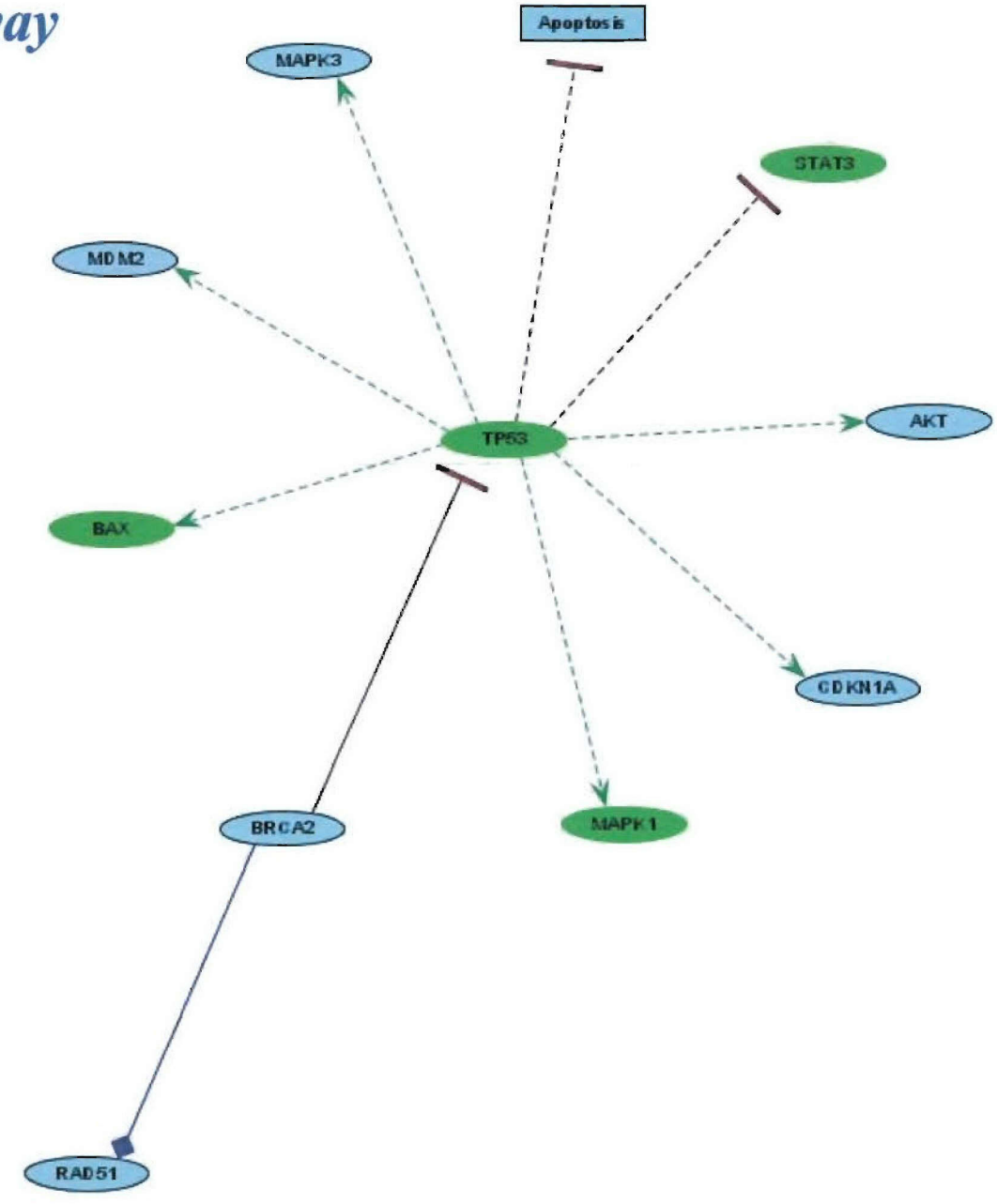

IGF signaling pathway
GSK3B
Cell Proliferation
BCAR1
PTYP
F-actin Disassembly
PTK2
ACTIN
IGFBP3
Depolarization of cells
PTPN11
IRS1
CDH1
PXN
IGF1R
CTNNA
PI3K
MAPK
IGF1
IGF1,IGF1R,TJP1
IRS2
TJP1
AKT2
MAPK1
MAPK3
IGF1,IGF1R
Cell-Cell Adhesion
Cell Survival
AKT
MAPK (P38)
Migration of cells
ESR1
PGR
TFF1
Related genes in this pathway
CDH1 – 20.66
PXN – 5.75
PTPN11 – 5.57 / 4.58
IGF1R – 3.14
MAPK1 – 2.6

Figure 5

p53 signaling pathway

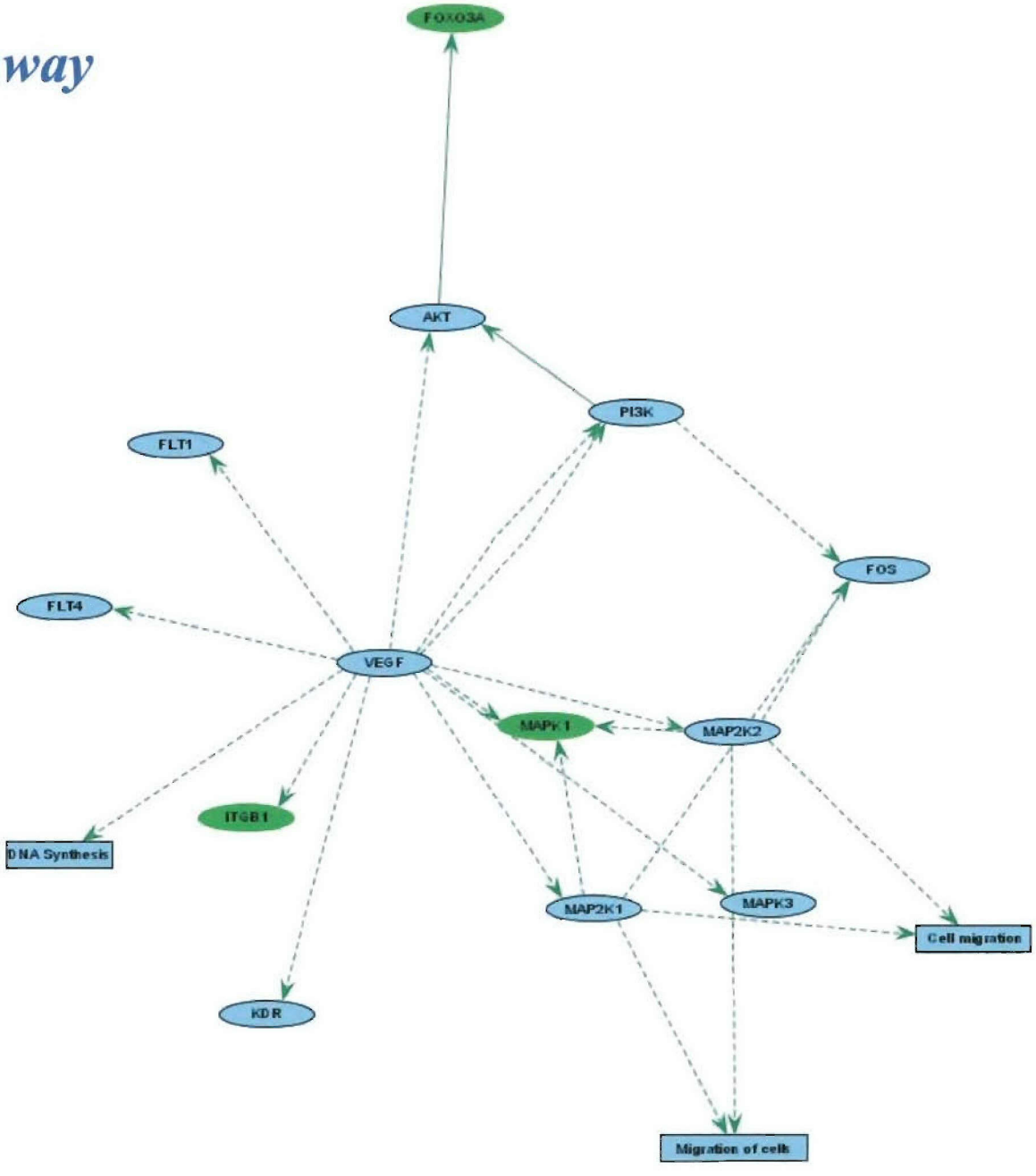


Related genes in this pathway

BAX – 3.65
TP53 – 3.03
STAT3 – 2.62
MAPK1 – 2.6

Figure 6

VEGF signaling pathway

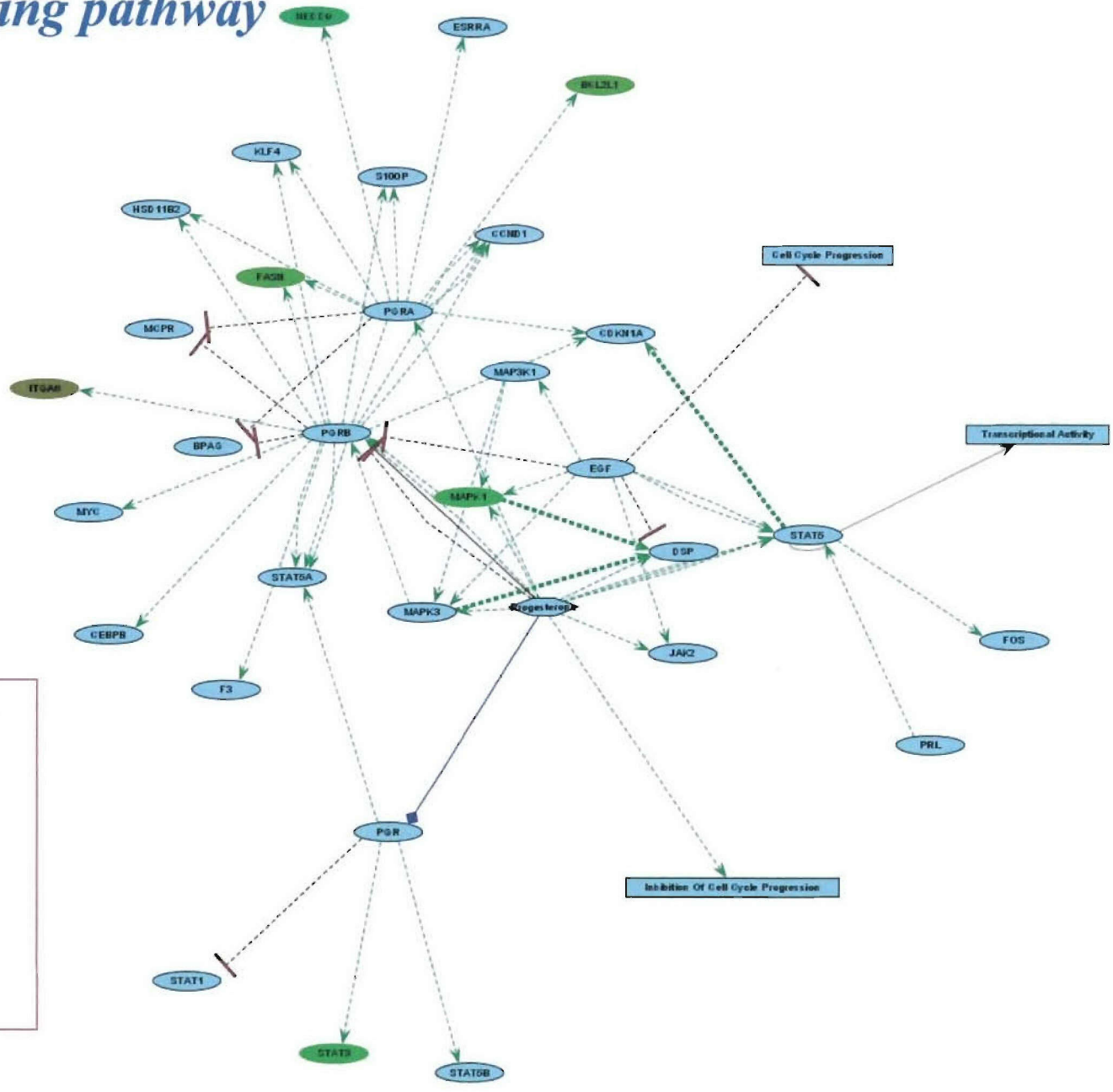


Related genes in this pathway

FOXO3A – 3.33
ITGB1 – 2.76
MAPK1 – 2.6

Figure 7

Progesterone signaling pathway



Related genes in this pathway

Integrin α6 – 10.97
BCL2L1 – 5.4
FASN – 5.15
STAT3 – 2.62
MAPK1 – 2.6
NEDD9 – - 3.39

Figure 8

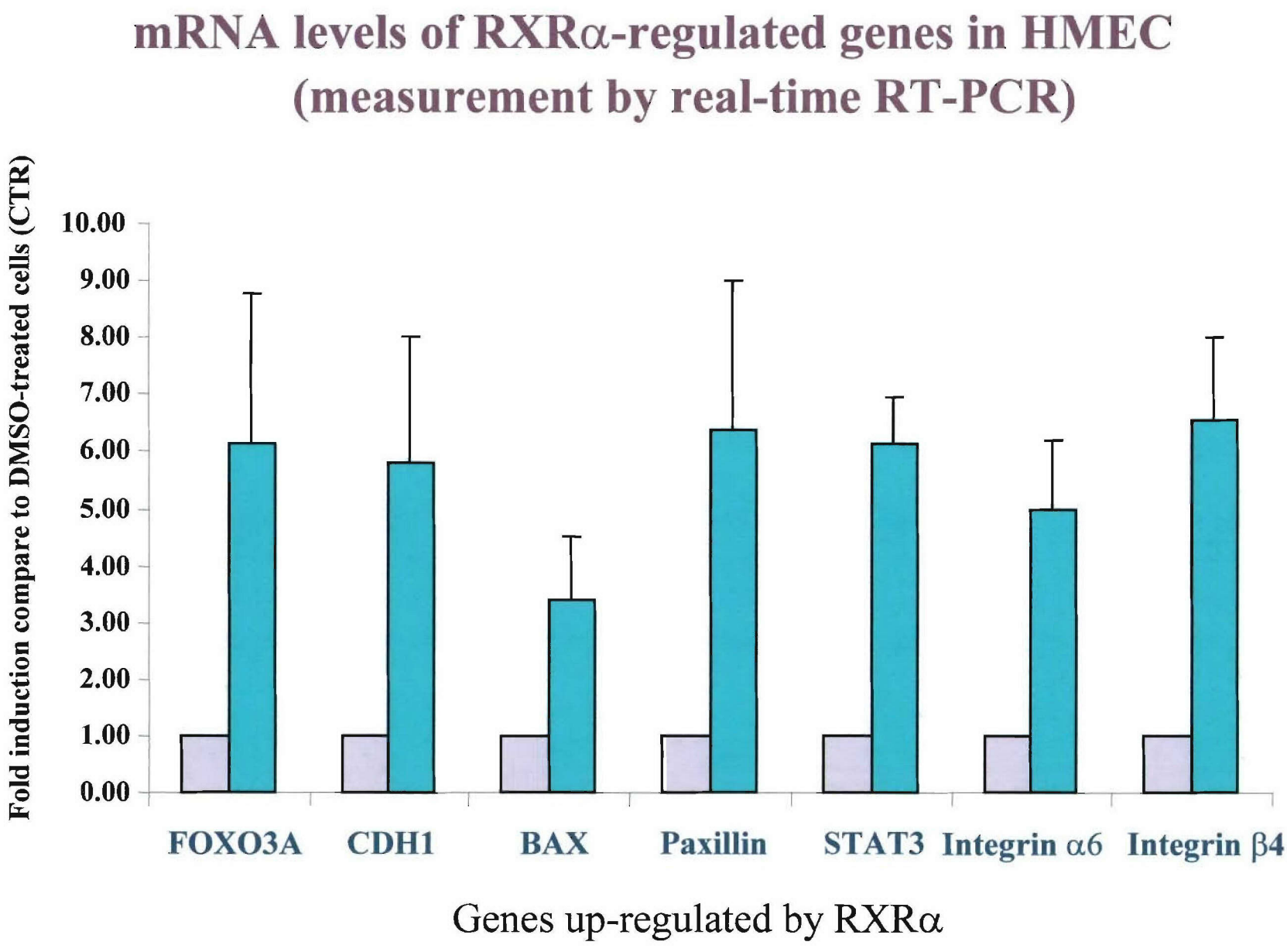

mRNA levels of RXRα-regulated genes in HMEC
(measurement by real-time RT-PCR)
Fold induction compare to DMSO-treated cells (CTR)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
FOXO3A
CDH1
BAX
Paxillin
STAT3
Integrin α6
Integrin β4
Genes up-regulated by RXRα

Figure 9

Protein levels of RXRα-regulated genes in HMEC (measurement by Western blot analysis)

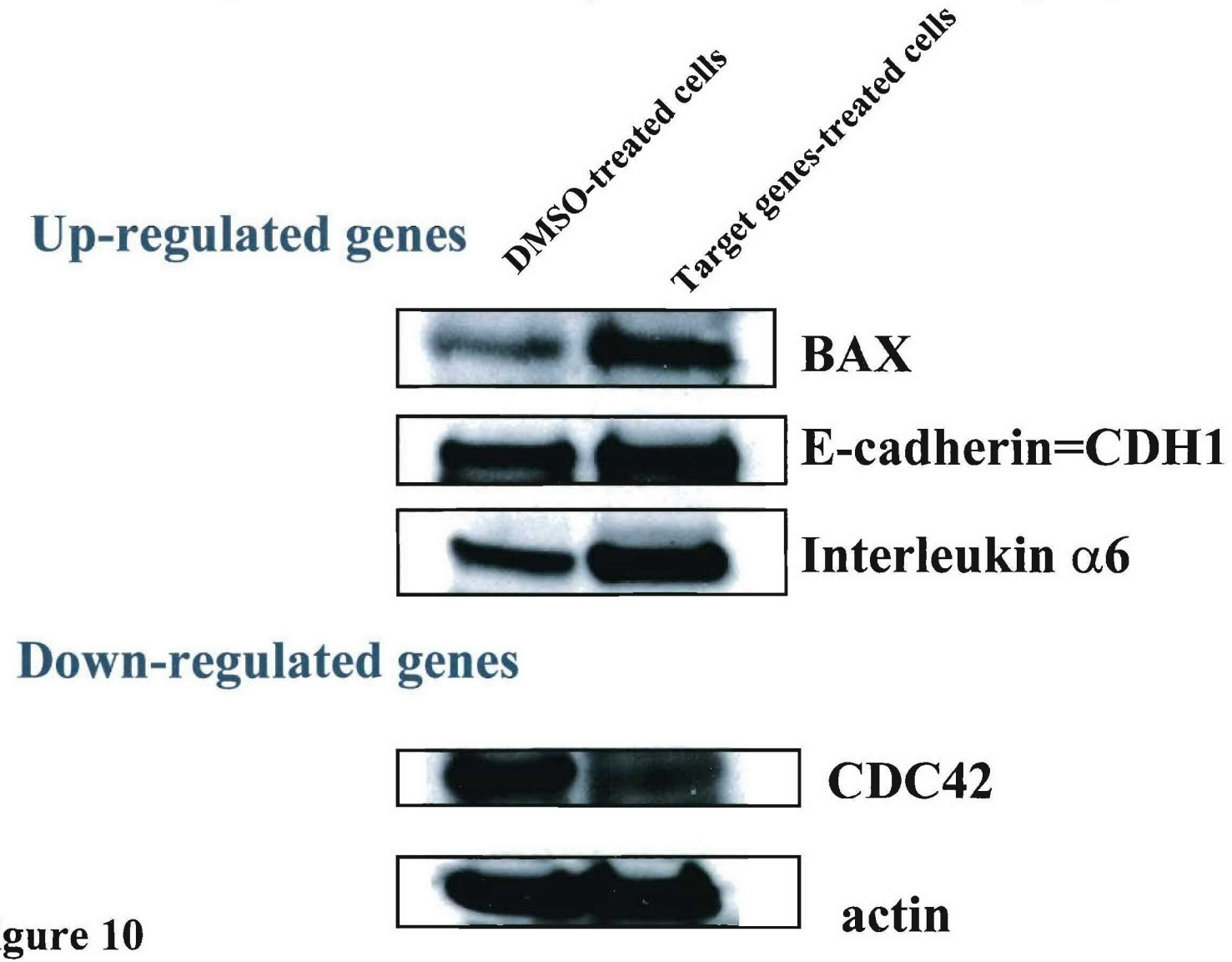


Figure 10

Table 3

Genes up-regulated by Ro25-7386 in MCF-7

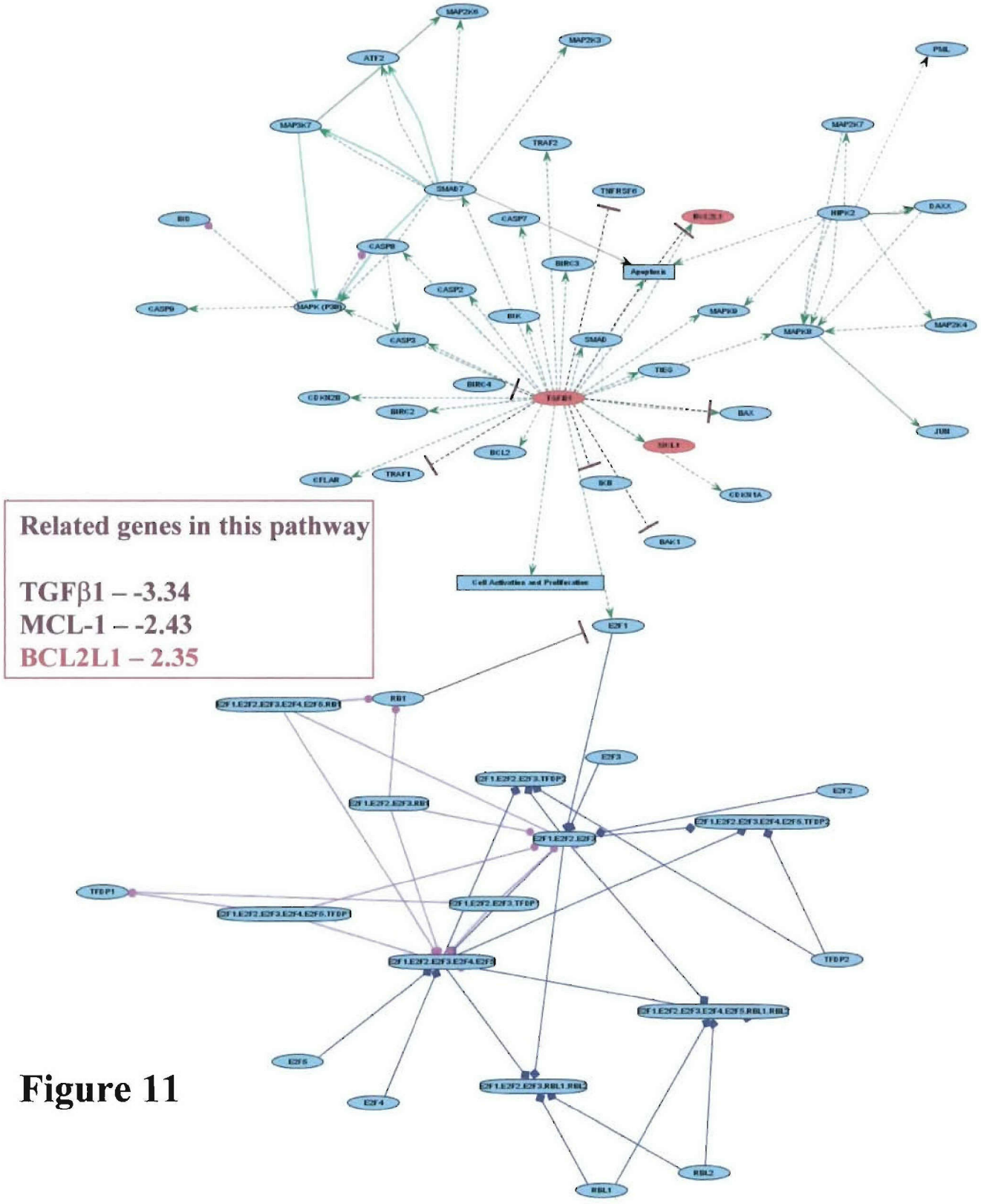
Probe Set	Gene Name	Fold Change	Gene Functions
209909_s_at	**transforming growth factor, beta 2**	**4.94**	**pivotal role in myocardial fibrosis and diastolic dysfunction, potent growth inhibitor in most epithelial cells**
213010_at	**protein kinase C, delta binding protein**	**3.76**	**superfamily of lipid-dependent protein Ser/Thr kinases**
63825_at	Abhydrolase domain containing 2	3.4	hydrolase activity
208993_s_at	peptidyl-prolyl isomerase G (cyclophilin G)	3.39	chaperone enzymes which alter the peptide bond between a given amino acid and a proline
204681_s_at	Rap guanine nucleotide exchange factor (GEF) 5	3.26	Guanine nucleotide exchange factor
213087_s_at	Eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	3.09	cadmium-responsive proto-oncogene
217489_s_at	interleukin 6 receptor	3.07	binding to IL-6, can produce anti-apoptotic effect and inflammation
205443_at	small nuclear RNA activating complex, polypeptide 1, 43kDa	3.04	required for transcription of SNRN genes by polymerases II and III
221815_at	Abhydrolase domain containing 2	2.95	hydrolase activity
205363_at	butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1	2.92	catalyzes the hydroxylation of gamma-butyrobetaine to carnitine, the last step in the biosynthesis of carnitine from lysine
210136_at	Myelin basic protein	2.88	one of the most important proteins of the myelin sheath
214255_at	ATPase, Class V, type 10A	2.87	putative aminophospholipid translocase
210841_s_at	neuropilin 2	2.82	a receptor for class III semaphorins and for certain members of the vascular endothelial growth factor family
204378_at	breast carcinoma amplified sequence 1	2.8	Amplified and over-expressed in breast cancer
201510_at	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	2.69	important regulator of morphogenesis and terminal differentiation of epithelial cell lineages in the small intestine
210089_s_at	laminin, alpha 4	2.68	the attachment, migration and organization of cells into tissues during embryonic development
208383_s_at	phosphoenolpyruvate carboxykinase 1 (soluble)	2.59	anaplerotic provision of carbon skeletons for amino acid biosynthesis in leaves of C3 plants
216657_at	ataxin 3	2.53	neurodegenerative disease-associated proteins
219737_s_at	protocadherin	2.52	synaptic function in the CNS
209225_x_at	transportin 1	2.36	protein transport, protein-nucleus import, docking,

Table 4

Genes down-regulated by Ro25-7386 in MCF-7

Probe Set	Gene Name	Fold Change	Gene Functions
202901_x_at	**cathepsin S**	**-64.37**	**lysosomal cysteine protease**
204989_s_at	integrin, beta 4	-4.51	cell-matrix adhesion, integrin-mediated signaling pathway
213042_s_at	ATPase, Ca++ transporting, ubiquitous	-4.42	control of the cytoplasmic free Ca2+ concentration and maintaining intracellular Ca2+ homeostasis
216971_s_at	plectin 1, intermediate filament binding protein 500kDa	-4.37	cytolinker protein, cytoskeletal regulator of PKC signaling
201167_x_at	Rho GDP dissociation inhibitor (GDI) alpha	–4.14	Rho protein signal transduction, negative regulation of cell adhesion
211905_s_at	integrin, beta 4	-3.87	cell-matrix adhesion, integrin-mediated signaling pathway
211672_s_at	actin related protein 2/3 complex, subunit 4, 20kDa	-3.7	crucial actin polymerization nucleator
207521_s_at	ATPase, Ca++ transporting, ubiquitous	-3.44	control of the cytoplasmic free Ca2+ concentration and maintaining intracellular Ca2+ homeostasis
207824_s_at	MYC-associated zinc finger protein (purine-binding transcription factor) (MAZ)	–3.42	DNA-binding protein, an activator, an initiator or a terminator of transcription
203085_s_at	**transforming growth factor, beta 1 (Camurati-Engelmann disease)**	**–3.34**	**anti-apoptosis, cell growth, cell proliferation**
203953_s_at	claudin 3	-3.26	formation of tight junctions
209872_s_at	plakophilin 3	-3.2	cell-cell adhesion
214326_x_at	jun D proto-oncogene	-3.14	nuclear component of the receptor tyrosine kinase/Ras signal transduction pathway
208677_s_at	**basigin (OK blood group)**	**–3.12**	**transmembrane protein involved in reproduction, neural function, inflammation and tumor invasion**
203751_x_at	jun D proto-oncogene	-3.08	nuclear component of the receptor tyrosine kinase/Ras signal transduction pathway
211823_s_at	paxillin	–2.93	cell motility, cell-matrix adhesion, signal complex formation
201373_at	plectin 1, intermediate filament binding protein 500kDa	–2.68	cytoskeletal anchoring, actin binding
218302_at	presenilin enhancer 2	–2.68	trigger presenilin endoproteolysis
213746_s_at	filamin A, alpha (actin binding protein 280)	-2.52	cell migration
200796_at	**myeloid cell leukemia sequence 1 (BCL2-related) (MCL-1)**	**–2.43**	**anti-apoptotic Bcl-2 family member**
206665_at	**BCL2-like 1(BCL2L1)**	**–2.35**	**apoptosis regulator Bcl-X**

TGFβ1-induced apoptosis



Related genes in this pathway

TGFβ1 – -3.34
MCL-1 – -2.43
BCL2L1 – 2.35

Figure 11

PTEN-mediated pathway

Related genes in this pathway

TGFβ2 – 4.94
BCL2L1 – 2.35

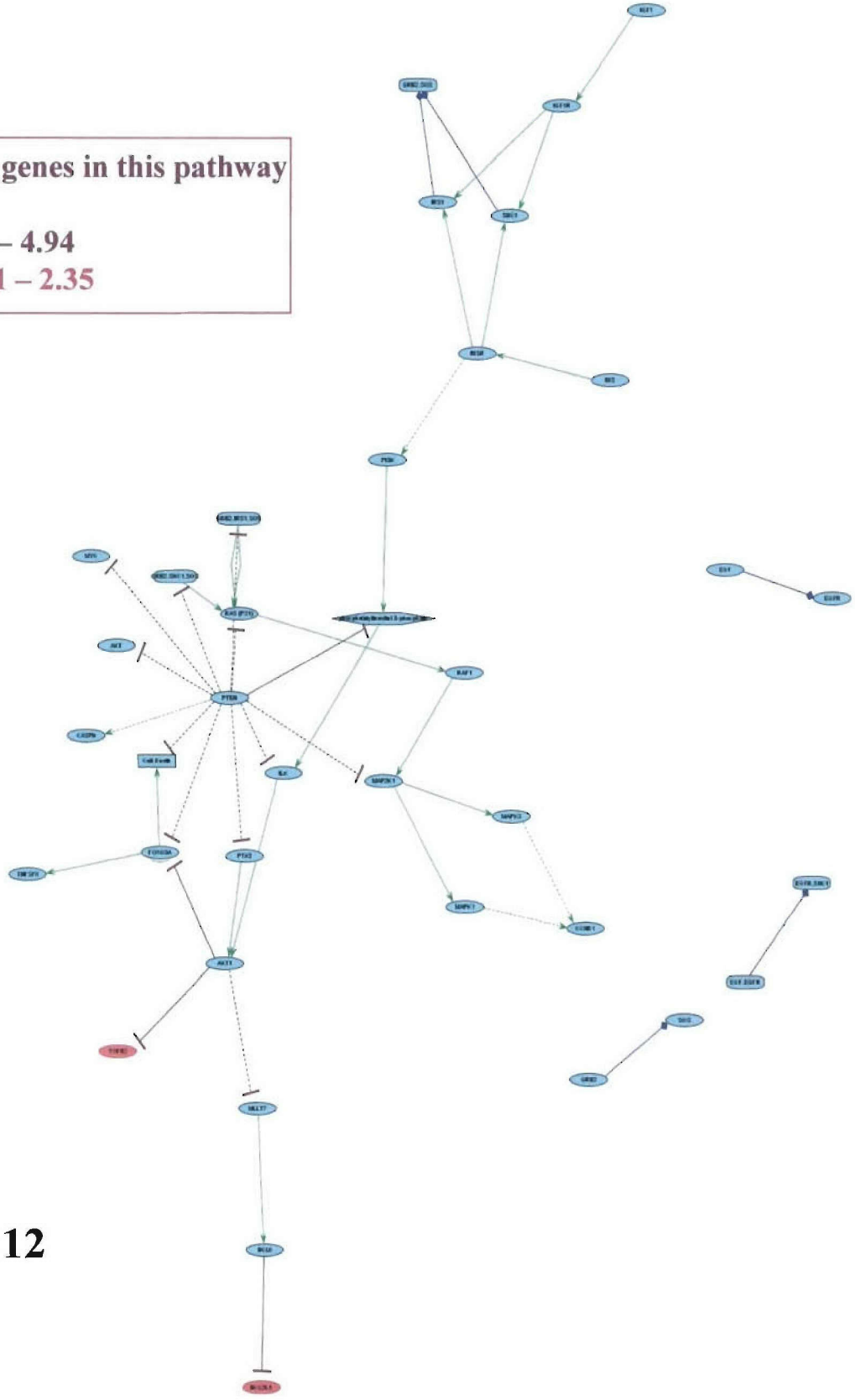


Figure 12

overexpressed in human breast cancer. Prior work in our lab showed that the down-regulation of overall levels of AIB1 plus Δ3AIB1, using a regulatable AIB1 directed ribozyme, resulted in reduced tumor growth *in vivo*. Overall, these data indicate a major role for AIB1 and its isoform Δ3AIB1 in breast cancer development and growth. However the relative roles of AIB1 versus the more highly active Δ3AIB1 in phenotypic changes in the breast has not been determined. As a prelude to these siRNA studies, we developed primers to specifically detect the Δ3AIB1 isoform by the technique of real-time PCR. The primer sequence specific for Δ3AIB1 overlaps the exon 3 splice junction. We used beta actin and GAPDH to normalize for RNA loading. Our results showed that for both the Δ3AIB1 plasmid control and the MCF-7 breast cancer cell line, we were able to pick up a signal for Δ3AIB1. This data provides a basis on which we can design siRNA to specifically target the Δ3AIB1 isoform. Ultimately, the information from this study will be used as a basis for the development of AIB1 and Δ3AIB1 directed siRNA as a potential therapy in humans.

The U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0344 supported this work.

P36-12: EVIDENCE THAT EMCA1 IS A GENETIC DETERMINANT OF E2-INDUCED MAMMARY TUMOR INCIDENCE AND TUMOR MULTIPLICITY IN THE ACI RAT

Beverly S. Schaffer,[1,2] Martin Tochacek,[2,3] Karen L. Pennington,[1,2] Jane L. Meza,[4] and James D. Shull[1,2,3,5]
[1]Department of Genetics, Cell Biology and Anatomy, [2]Eppley Institute for Cancer Research, [3]Department of Biochemistry and Molecular Biology, [4]Department of Preventive and Societal Medicine, [5]Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE
E-mail: bschaffe@unmc.edu

When treated with 17β estradiol (E2), female ACI rats rapidly develop mammary cancers. However, Brown Norway (BN) rats are resistant to E2-induced mammary cancers. Linkage analysis of F2 progeny from reciprocal crosses between ACI and BN rat strains identified seven loci that determine susceptibility to E2-induced mammary carcinogenesis. Emca1 (Estrogen-induced mammary cancer 1) localizes to Chromosome 5 (RNO5) and spans approximately 95.1 Mb between polymorphic markers D5Rat134 and D5Rat171. To determine the effect of Emca1 in vivo, a 136.4 Mb region of the BN RNO5, from polymorphic marker D5Rat190 through D5Rat205 encompassing the Emca1 locus, was introgressed onto an ACI background to produce the ACI.BN-D5Rat190:D5Rat205 congenic strain. Female ACI.BN- D5Rat190:D5Rat205 rats (n = 41) rats treated with E2 for 28 weeks exhibited significantly reduced incidence of mammary cancer ($P \leq 0.01$; 66%) versus ACI rats (90%) treated with E2 for 28 weeks. Latency to the development of the first palpable tumor was significantly ($P \leq 0.0001$) prolonged in the ACI.BN- D5Rat190:D5Rat205 rats (171.5 ± 3.9 days) compared to ACI rats (136.0 ± 3.5 days). Tumor burden was also significantly ($P < 0.05$) reduced in the ACI.BN- D5Rat190:D5Rat205 rats (1.98 ± 0.2 tumors per rat) compared to the ACI rats (4.96 ± 0.6 tumors per rat). We have developed three additional congenic lines, in which a region of Emca1 from the BN genome on RNO5 has been introgressed onto an ACI background, and evaluation of the effect of E2 on the development of mammary tumors in these congenic lines is currently underway. We have also developed a fourth congenic line in which an 8.5 Mb region of the BN genome that includes the cdkn2a gene, a candidate gene for the Emca1 locus, has been introgressed onto an ACI background. Evaluation of the effect of E2 on this congenic line is also underway.

The U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0477 supported this work.

P36-13: IDENTIFICATION OF RXR ALPHA TARGET GENES THAT ARE INVOLVED IN THE SUPPRESSION OF THE GROWTH IN HUMAN MAMMARY EPITHELIAL CELLS (HMEC)

Hye-Sook Seo, Kevin R. Coombes, and Ja-Seok Koo
Department of Thoracic/Head and Neck Medical Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX
E-mail: hseo@mdanderson.org

Retinoids, the natural and synthetic derivatives of vitamin A, modulate gene expression or function related to embryonal development, cellular proliferation, differentiation, and apoptosis via two finctionally different receptors, retinoid acid receptor (RAR) and retinoid X receptor (RXR). Retinoids are also known by their ability to inhibit tumor growth and metastasis in breast cancer indicating their promising chemo-preventive and therapeutic property. Especially, RXR-bound compounds (rexinoid) suppress ER-positive and ER-negative mammary tumors with reduced toxicity compared to RAR-bound compounds (Gottardis et al., 1996; Wu et al., 2002). Among RXR receptor isoforms α, β, and γ, RXR α seems to play a critical role in tumor suppression (Crowe and Chandraratna, 2004). Thus, we focussed our interest on the α isoform using an RXR α specific ligand, Ro25-7328, to study whether this rexinoid suppresses breast cell growth. For that purpose we first tested the ability of Ro25-7328 to inhibit the cell growth in normal HMEC and the retinoid-sensitive breast cancer cell line T47D. By MTT assay, we found that the RXR α agonist, Ro25-7328 suppressed the growth of both HMEC and T47D by a dose-dependent manner.

To identify genes that are regulated by RXR α, we treated HMEC cells with Ro25-7328 for 12h using the dose which most strongly suppressed the cell growth (10 μM). We then examined changes in gene expression by using the affymetrix microarray (human genome U133A 2.0) to investigate which genes are related to cell growth inhibition induced by the RXR α agonist. In HMEC we identified 638 genes up-regualted and 347 genes down-regulated by Ro25-7328 with changes in fold induction greater than 2 fold. 22 genes were strongly up-regulated (more than 10 fold), and 5 genes were strongly down-regulated (more than 4 fold) in expression levels by Ro25-7328. Among them, we found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus; i.e., integrin β 4, integrin α 6, fibronectin 1, BCL2-associated X protein (BAX), β 5 tublin, forkhead box O3A (FOXO3A), collagen type VI α 3, cell division cycle 42 (CDC42), chemokine ligand 14 (CXCL14) etc. Further focussing on the implication of those genes in Ro25-7328-induced growth suppression we seek to clarify the mecahnism by which rexinoids suppress breast cancer development.

In conlcusion we found from this study, that RXR α agonist Ro25-7328 suppressed breast cell growth (normal breast cells, HMEC and retinoid-sensitive breast cancer cells, T47D). We also identified a set of genes which may be involved in cell growth inhibition regulated by Ro25-7328 using the affymetrix microarray.

The U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0505 supported this work.

P36-14: ANALYSIS OF PROGESTIN EFFECTS ON HEPATOCYTE GROWTH FACTOR SIGNALING PATHWAYS IN RELATION TO PROLIFERATION AND ALVEOLAR MORPHOGENESIS OF NORMAL MAMMARY EPITHELIAL CELLS IN VITRO

Kyle T. Smith and Sandra Z. Haslam, Ph.D.
Cell and Molecular Biology Program and Department of Physiology and Human Medicine, Michigan State University, East Lansing, MI
E-mail: smithky7@msu.edu

Progestins (P) are major mitogens in the adult human breast and can significantly contribute to breast cancer risk. To study P action we have developed a serum free, three dimensional system to study mammary organoids in primary culture. We determined that for P-induced proliferation and morphogenesis to occur in normal mouse mammary cells, the presence of hepatocyte growth factor (HGF) is required. HGF alone induces proliferation and a ductal morphogenesis. Treatment with HGF+P results in increased proliferation and alveolar-like morphogenesis.

The goal of this study was to determine the signaling pathways by which P and HGF interact to promote growth of normal adult mammary epithelial cells. This was accomplished by use of immunohistochemical staining of HGF signaling intermediates and biochemical inhibitors of relevant signaling pathways. The expression of Met was examined after treatments of HGF or HGF+P to determine if P altered HGF signaling at the level of the receptor. No difference in Met expression was seen under HGF+P treatment, however it was noted that Met expression in the myoepithelial cells was higher than the luminal cells. The expression of cyclin D1, required for alveologenesis in vivo, was also investigated. Cyclin D1 was more highly expressed under HGF+P treatment and was localized primarily to the nucleus compared to HGF or P alone. Progesterone receptor (PR) isoform expression was examined since it has been shown that the ratio of the A and B isoforms can affect alveolar formation. PR A expression appeared to be down-regulated by HGF+P versus other treatments. PR B was most highly expressed in organoids treated with HGF+P. Since both the extra-cellular regulated kinase (ERK) and the phosphatidyl-inositol 3-kinase (PI3K) pathways have been shown to be important for HGF induced proliferation and morphogenesis, inhibitors of those pathways were used. Blocking of either mitogen/extracellular signal-regulated kinase kinase (MEK1/2) or PI3K resulted in a 50% reduction in proliferation of HGF and HGF+P treated organoids. These inhibitors had less inhibitory effect on the ductal and alveolar- like morphologies produced by HGF or HGF+P treatment respectively.

We conclude that increased expression of cyclin D1 and PR B are correlated with increased proliferation and alveolar-like morphology seen in response to HGF+P treatment. It also appears that the PI3K and MEK1/2 signaling intermediates in the pathways responsible for the proliferative and morphologic responses of HGF+P. It is expected that the understanding of P-induced proliferation in normal mammary epithelial cells will advance our understanding of the role of P in breast cancer development.

The U.S. Army Medical Research and Materiel Command under DAMD17-02-1-0488 supported this work.

Identification of RXR alpha target genes that are involved in the suppression of the growth in human mammary epithelial cells

Department of Thoracic/Head and Neck Medical Oncology
University of Texas M. D. Anderson Cancer Center
Houston, Texas

Hye-Sook Seo, Kevin R. Coombes and Ja-Seok Koo

ABSTRACT

Retinoids, the natural and synthetic derivatives of vitamin A, modulate gene expression or function related to embryonal development, cellular proliferation, differentiation, and apoptosis via two finctionally different receptors, retinoid acid receptor (RAR) and retinoid X receptor (RXR). Retinoids are also known by their ability to inhibit tumor growth and metastasis in breast cancer indicating their promising chemo-preventive and therapeutic property. Especially, RXR-bound compounds (rexinoid) suppress ER-positive and ER-negative mammary tumors with reduced toxicity compared to RAR-bound compounds (Gottardis et al., 1996; Wu et al., 2002). Among RXR receptor isoforms alpha, beta, and gamma, RXR alpha seems to play a critical role in tumor suppression (Crowe and Chandraratna, 2004). Thus, we focused our interest on the alpha isoform using an RXR alpha specific ligand, Ro25-7328, to study whether this rexinoid suppresses breast cell growth. For that purpose we first tested the ability of Ro25-7328 to inhibit the cell growth in normal HMEC and the retinoid-sensitive breast cancer cell line T47D. By MTT assay, we found that the RXR alpha agonist, Ro25-7328 suppressed the growth of both HMEC and T47D by a dose-dependent manner.

To identify genes that are regulated by RXR alpha, we treated HMEC cells with Ro25-7328 for 12h using the dose which most strongly suppressed the cell growth (10 uM). We then examined changes in gene expression by using the affymetrix microarray (human genome U133A 2.0) to investigate which genes are related to cell growth inhibition induced by the RXR alpha agonist. In HMEC we identified 638 genes up-regualted and 347 genes down-regulated by Ro25-7328 with changes in fold induction greater than 2 fold. 22 genes were strongly up-regulated (more than 10 fold), and 5 genes were strongly down-regulated (more than 4 fold) in expression levels by Ro25-7328. Among them, we found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus; i.e., integrin beta 4, integrin alpha 6, fibronectin 1, BCL2-associated X protein (BAX), beta 5 tublin, forkhead box O3A (FOXO3A), collagen type VI alpha 3, cell division cycle 42 (CDC42), chemokine ligand 14 (CXCL14) etc. Further focusing on the implication of those genes in Ro25-7328-induced growth suppression we seek to clarify the mecahnism by which rexinoids suppress breast cancer development.

In conclusion, we found from this study, that RXR alpha agonist Ro25-7328 suppressed breast cell growth (normal breast cells, HMEC and retinoid-sensitive breast cancer cells, T47D). We also identified a set of genes which may be involved in cell growth inhibition regulated by Ro25-7328 using the affymetrix microarray.

INTRODUCTION

Retinoids regulate a variety of biological functions such as embryogenesis, growth, differentiation, vision and reproduction, and play important roles in modulating normal- or tumor-cell growth through the regulation of differentiation and/or apoptosis, thus supporting the potential use of retinoids in cancer therapy and/or prevention (Niles, 2000; Zusi et al., 2002). In addition to naturally occurring retinoids, such as all-*trans* retinoic acid (ATRA), 9-*cis* retinoic acid (9-*cis*-RA) and 13-*cis* retinoic acid (13-*cis*-RA), various synthetic retinoids with different selectivity have been developed and are currently available to treat acne, psoriasis, and actinic keratosis or cancers such as acute promelocytic leukemia, cutaneous T-cell lymphoma, and squamous or basal cell carcinoma (Dawson, 2004). The retinoids exert their anti-proliferative effects in target cells interacting with RARs and RXRs, each of which comprise three isotypes designated α, β and γ.

RXR-selective retinoids (rexinoids) are important in controlling apoptosis and can function in a ligand-dependent or ligand-independent manner (Nagy et al., 1998; Lippman and Lotan, 2000). RXR are well-known to heterodimerize with several steroid hormone receptors, such as RAR, TR, VDR, PPAR, LXR, PXR and FXR suggesting its involvement in several signaling pathways. RXR also homodimerize in transfected cells (Zusi et al., 2002). RXR agonists have shown more active and less skin toxic property than RAR-selective ligands in animal models of breast cancer prevention studies (Gottardis et al., 1996; Lippman and Lotan, 2000; Wu et al., 2002). Rexinoids are also active in animals in tamoxifen-resistant breast cancer (Bischoff et al., 1999; Lippman and Lotan, 2000) and in ATRA-resistant breast cancer cells (Crowe and Chandraratna, 2004) suggesting its prominent chemo-preventivetherapeutic activity.

We, therefore, are interested in RXR function in breast cells with their regulatory activity in transcription of genes involved in growth suppression. We concentrated our focus on the RXRα isoform which seems to be a potential therapeutic target in breast cancer cells since over-expression of RXR α sensitized breast cancer cells lines to the anti-proliferative effect of RXR-selective ligand (Crowe and Chandraratna, 2004), moreover, infection of adenoviral RXRα induced nucleoplasmic over-expression of RXRα and resulted in apoptosis under treatment with an RXR ligand in retinoid-resistant MDA-MB-231 (Tanaka et al., 2004). Hence, in this study, we tested 1) the growth suppressive activity of RXRα specific ligand, Ro25-7328 in normal breast cells (HMEC) and retinoid-sensitive breast cancer cells (T47D) by MTT assay, and then, 2) identified genes regulated by RXRα which may be involved in the anti-proliferative activity of Ro25-7328. For that purpose, we treated HMEC with Ro25-7328 for 12h using the dose which most strongly suppressed the cell growth (10 uM). We then examined changes in gene expression by using the affymetrix microarray (human genome U133A 2.0) to investigate which genes are related to cell growth inhibition induced by the RXRα agonist. In HMEC, we identified a variety of genes which are involved in cell apoptosis, cell growth/maintenance, signal transduction, response to stimulus and breast cancer. We confirmed their expression levels in fold induction changes by real-time RT-PCR and western blot analysis. Our further purpose of this study is the investigation of selecting genes more thoroughly to clarify their implication in HMEC cell growth suppression induced by Ro25-7328, thus achieving our ultimate goal to find the mechanism by which retinoids suppress breast cancer development.

Primary amino sequences of RARs and RXRs (Okuno et al., 2004)

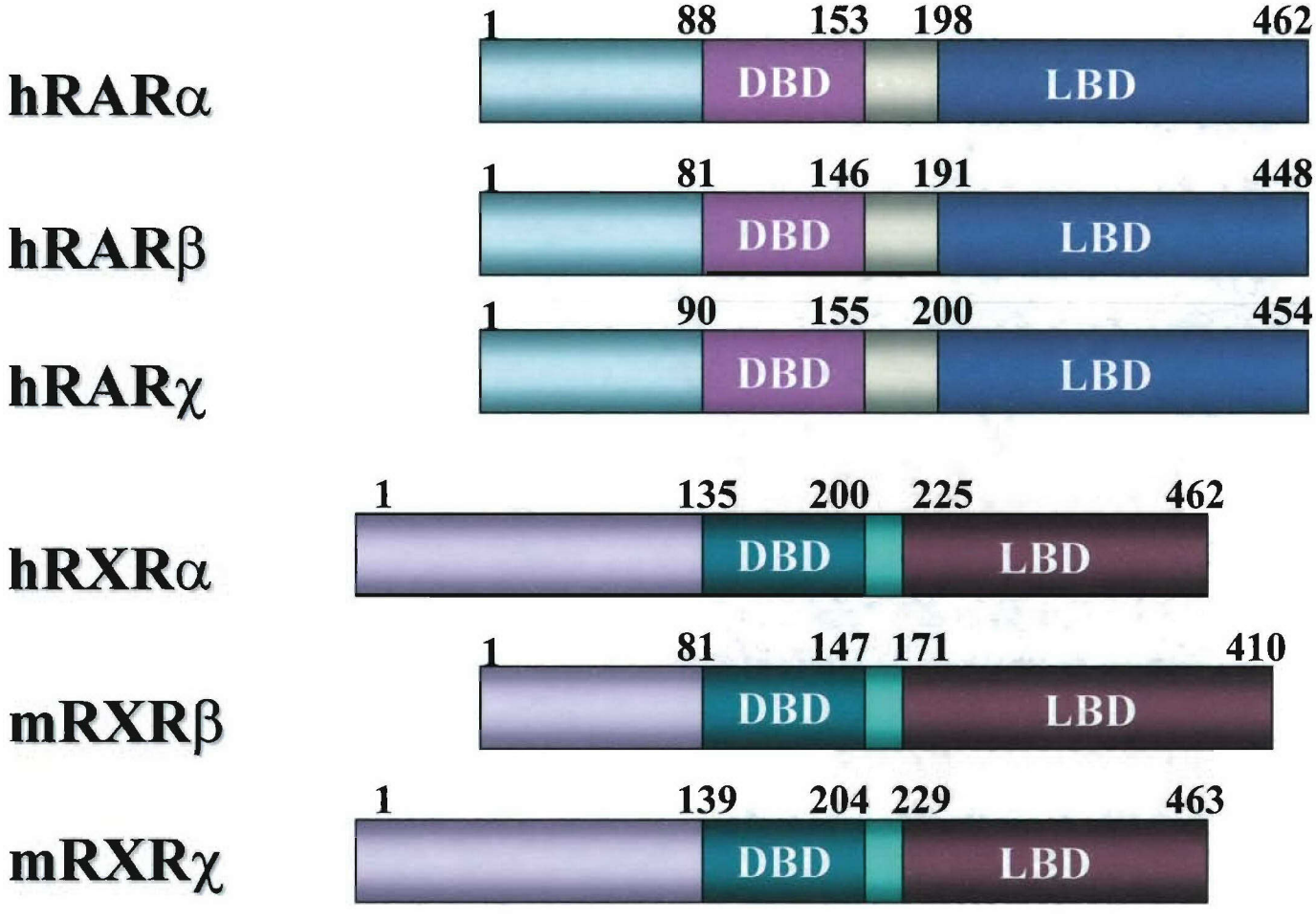
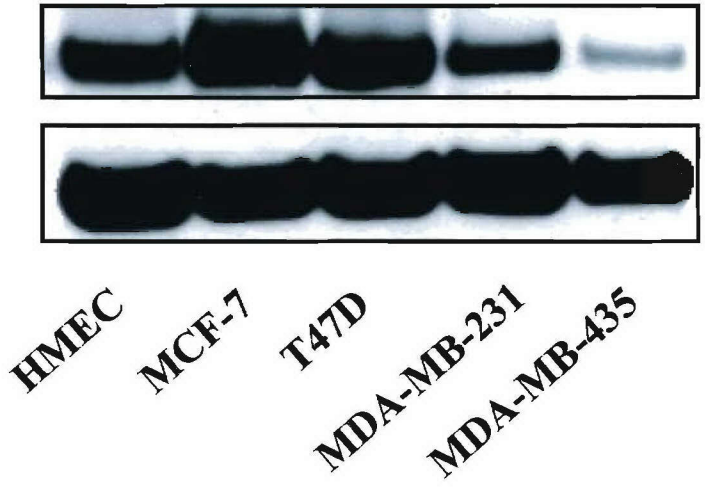


Figure 1

RESULT

Expression of RXR alpha in breast cells (western blot analysis) - normal (HMEC) and breast cancer cells



Normal Human Mammary Epithelial cells (HMEC), retinoid-sensitive breast cancer cells (T47D and MCF-7) and retinoid-insensitive breast cancer cells (MDA-MB-231 and MDA-MB-435) express RXR alpha.

Figure 2

1. ***RXR alpha agonist, Ro25-7325 suppresses HMEC cell growth by dose-dependent manner. All-trans RA, 9-cis-RA and 13-cis-RA also have the tendency to inhibit cell growth.***

2. ***RXR alpha agonist, Ro25-7325 suppresses T-47D cell growth. All-trans RA, 9-cis-RA and 13-cis-RA strongly inhibit the cell growth.***

Effect of RXR alpha agonist Ro25-7325 on HMEC normal breast cell growth (MTT assay)

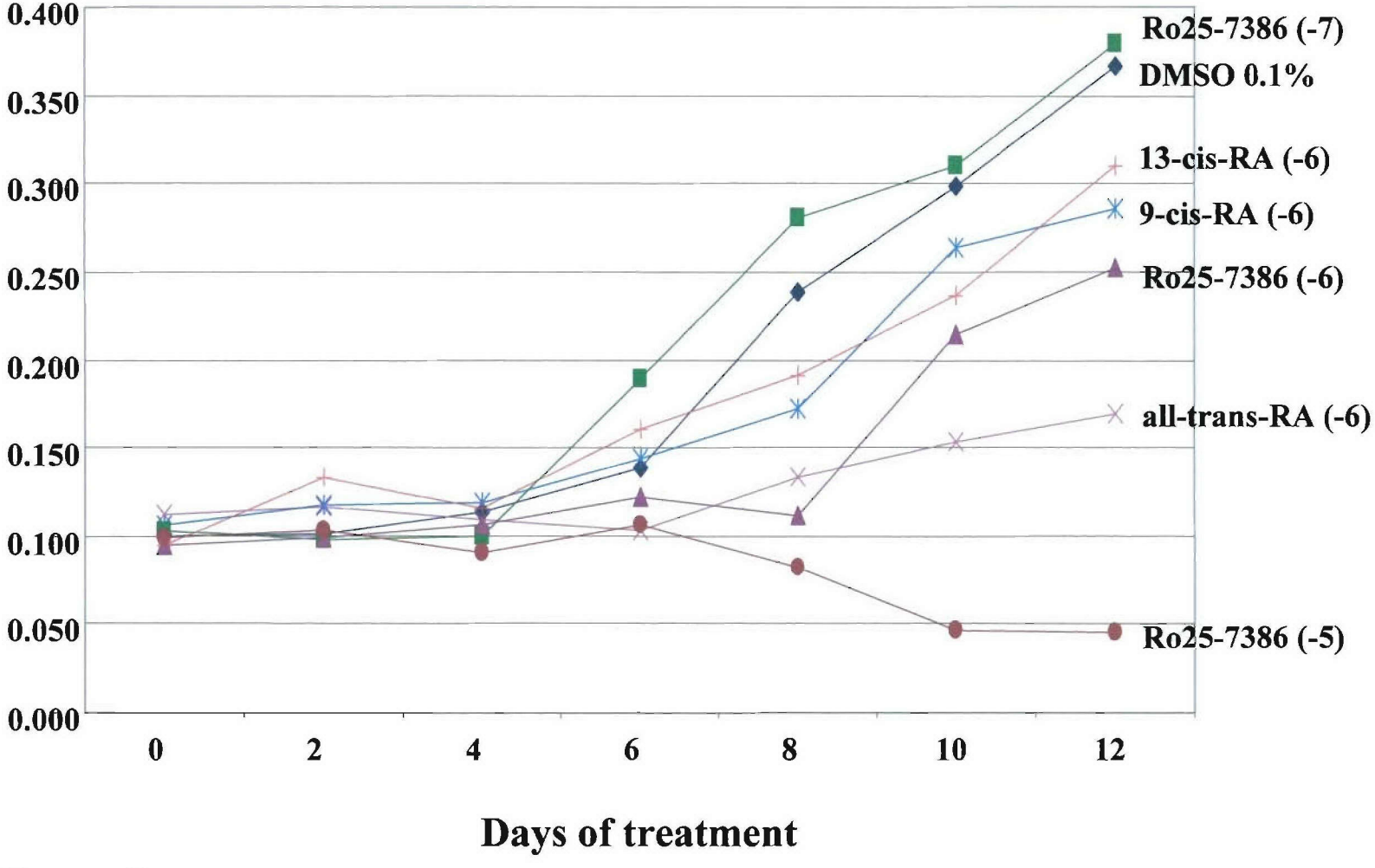


Figure 3

Effect of RXR alpha agonist Ro25-7325 on T47D breast cancer cell growth (MTT assay)

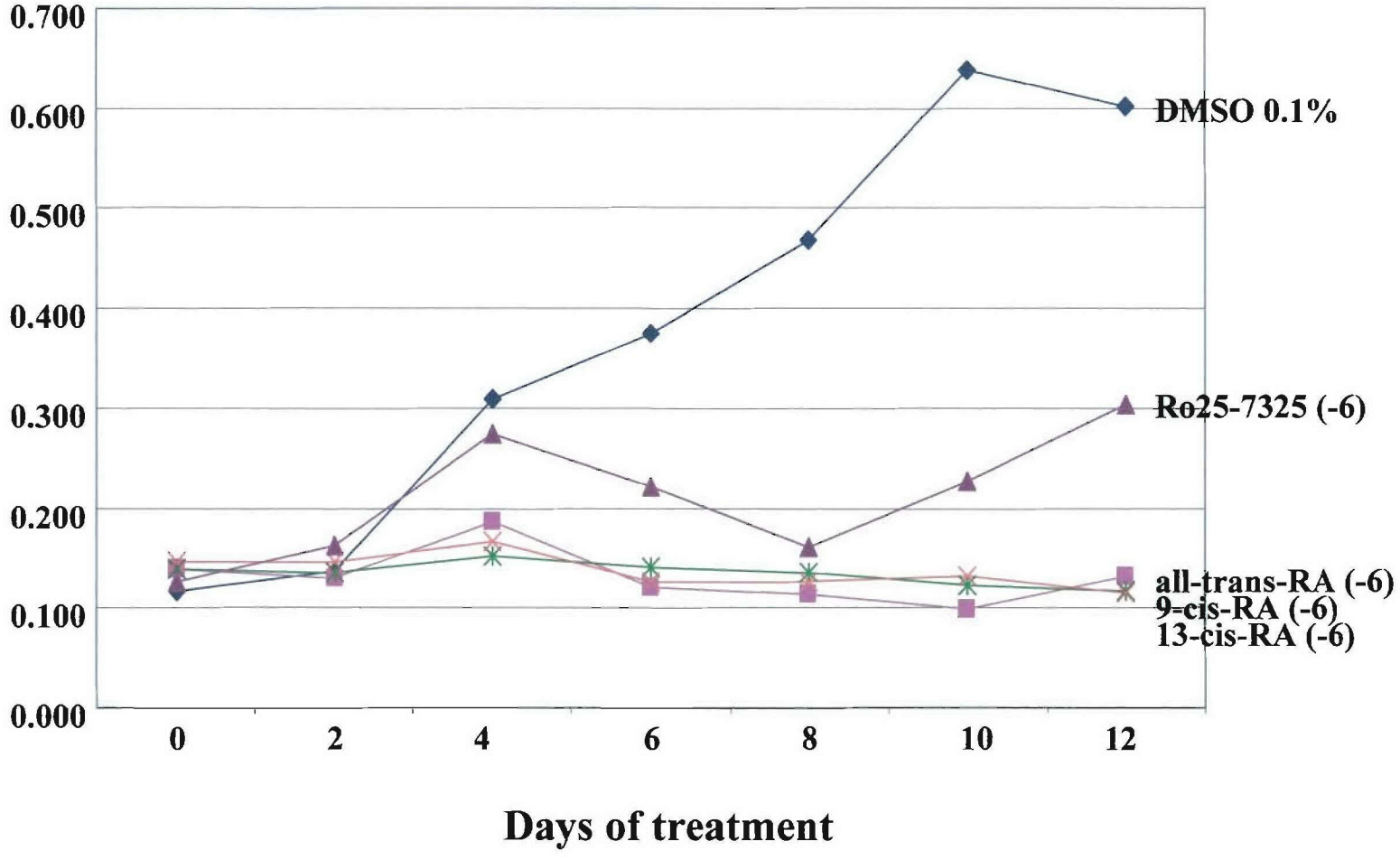


Figure 4

By using the affymetrix microarray (human genome U133A 2.0), we found 638 genes up-regulated and 347 genes down-regulated by Ro25-7328 with changes in fold induction greater than 2 fold in the HMEC normal breast cell line - 22 genes were strongly up-regulated (more than 10 fold), and 5 genes were strongly down-regulated (more than 4 fold) in expression levels by Ro25-7328. We selected our major genes of interest by referring to PathArt program (see Figure 5-8).

Table 1

Genes up-regulated by Ro25-7386 in HMEC

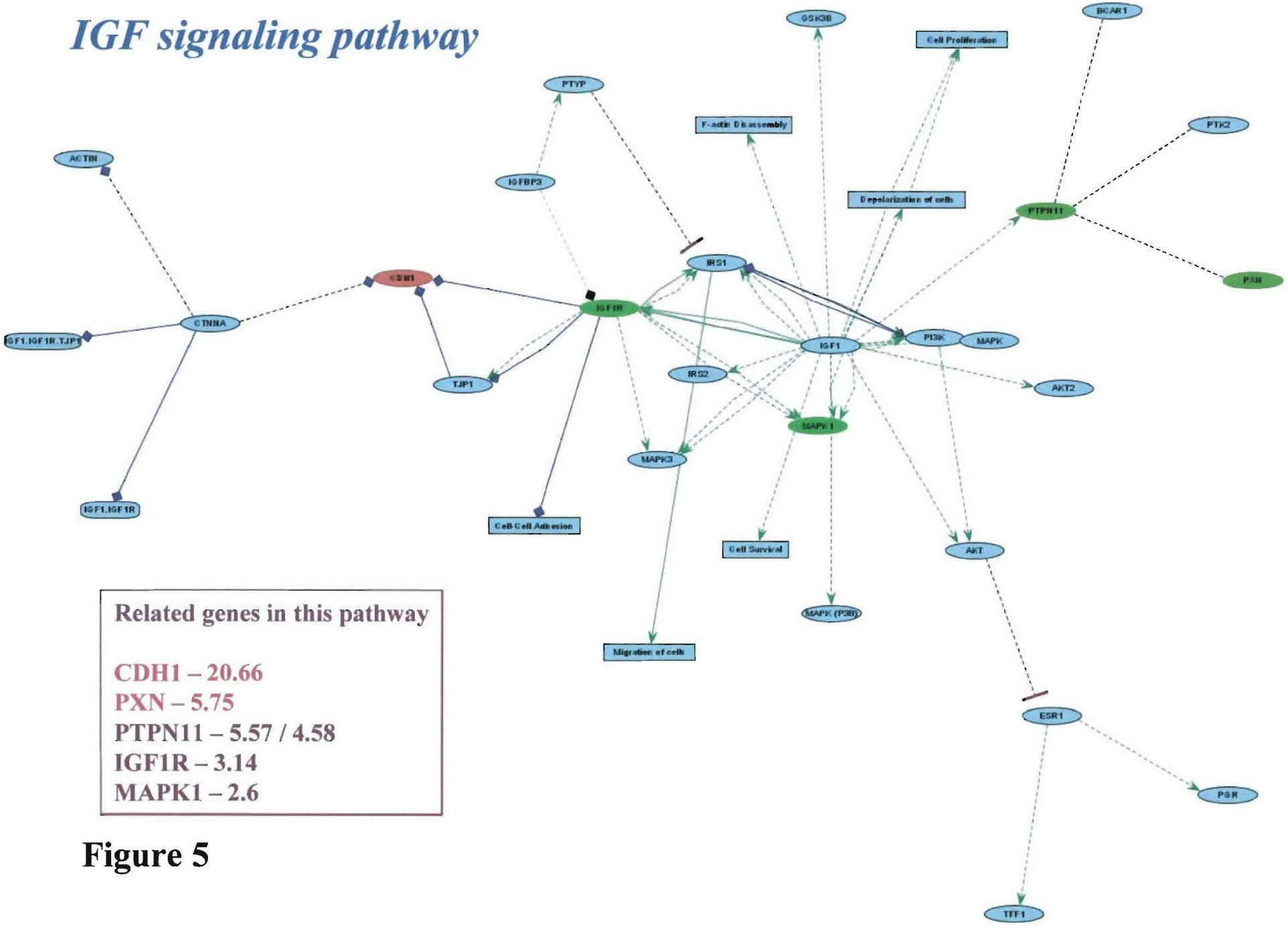
Probe Set	Gene Name	Fold Change	Gene Functions
204989_s_at	**integrin, beta 4**	**26.6**	**cell-matrix adhesion, integrin-mediated signaling pathway**
201130_s_at	**cadherin 1, type 1, E-cadherin (epithelial) (CDH1)**	**20.66**	**cell-to-cell adhesion protein, potent invasion/tumor suppressor**
203392_s_at	C-terminal binding protein 1	16.35	L-serine biosynthesis, negative regulation of cell proliferation
216971_s_at	plectin 1, intermediate filament binding protein 500kDa	15.17	cytoskeletal anchoring
215780_s_at	SET translocation (myeloid leukemia-associated)	12.51	DNA replication, negative regulation of histone acetylation
201971_s_at	ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A	11.75	ATP synthesis coupled proton transport, proton transport
204426_at	coated vesicle membrane protein	11.74	intracellular protein transport
215177_s_at	**integrin, alpha 6**	**10.97**	**cell-matrix adhesion, cell-substrate junction assembly**
211905_s_at	**integrin, beta 4**	**10.34**	**cell-matrix adhesion, integrin-mediated signaling pathway**
201048_x_at	RAB6A, member RAS oncogene family	10.03	protein transport, small GTPase mediated signal transduction
214701_s_at	fibronectin 1	10.01	acute-phase response, cell adhesion, cell migration, metabolism
202118_s_at	copine III	9.48	lipid metabolism, vesicle-mediated transport
217234_s_at	villin 2 (ezrin)	9.09	cellular morphogenesis, cytoskeletal anchoring
202855_s_at	solute carrier family 16 (monocarboxylic acid transporters), member 3	9.06	monocarboxylic acid transport, transport
210935_s_at	WD repeat domain 1	8.44	perception of sound
208750_s_at	ADP-ribosylation factor 1	7.31	intracellular protein transport
202856_s_at	solute carrier family 16 (monocarboxylic acid transporters), member 3	7.26	monocarboxylic acid transport, transport
200796_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	7.25	cell differentiation, regulation of apoptosis
213606_s_at	Rho GDP dissociation inhibitor (GDI) alpha	7.25	Rho protein signal transduction, negative regulation of cell adhesion
201373_at	plectin 1, intermediate filament binding protein 500kDa	7.19	cytoskeletal anchoring
208057_s_at	GLI-Kruppel family member GLI2	7.04	organogenesis, regulation of transcription-DNA-dependent
217294_s_at	enolase 1, (alpha)	6.99	glycolysis, negative regulation of cell growth
200806_s_at	heat shock 60kDa protein 1 (chaperonin)	6.69	mitochondrial matrix protein import, protein folding
211823_s_at	**paxillin (PAX)**	**5.75**	**cell motility, cell-matrix adhesion, signal complex formation**
211833_s_at	**BCL2-associated X protein (BAX)**	**3.65**	**apoptosis, negative regulation of cell cycle**
210655_s_at	**forkhead box O3A (FOXO3)**	**3.33**	**apoptosis, cell growth and/or maintenance**
208992_s_at	**signal transducer and activator of transcription 3 (STAT3)**	**2.62**	**cell motility, intracellular signaling cascade, neurogenesis**

Table 2

Genes down-regulated by Ro25-7386 in HMEC

Probe Set	Gene Name	Fold Change	Gene Functions
201438_at	**collagen, type VI, alpha 3**	**-4.59**	**cell adhesion, muscle development, phosphate transport**
204634_at	NIMA (never in mitosis gene a)-related kinase 4	-3.59	mitosis, protein amino acid phosphorylation
209788_s_at	type 1 tumor necrosis factor receptor shedding aminopeptidase regulator	-3.48	adipocyte differentiation, antigen processing
205802_at	transient receptor potential cation channel, subfamily C, member 1	-3.43	calcium ion transport, cation transport
202149_at	neural precursor cell expressed, developmentally down-regulated 9	-3.39	actin filament bundle formation, regulation of cell cycle
204415_at	interferon, alpha-inducible protein (clone IFI-6-16)	-3.29	immune response, response to pest, pathogen or parasite
210017_at	mucosa associated lymphoid tissue lymphoma translocation gene 1	-3.12	activation of NF-kappaB-inducing kinase, anti-apoptosis
205420_at	peroxisomal biogenesis factor 7	-3.05	protein transport
219317_at	polymerase (DNA directed) iota	-3.01	DNA repair
204176_at	kelch-like ECT2 interacting protein	-3	cytoskeleton organization and biogenesis
203741_s_at	adenylate cyclase 7	-2.95	cAMP biosynthesis, intracellular signaling cascade
204078_at	synaptonemal complex protein SC65	-2.9	synaptonemal complex formation
203881_s_at	dystrophin (muscular dystrophy, Duchenne and Becker types)	-2.88	cytoskeletal anchoring, muscle contraction
209717_at	ecotropic viral integration site 5	-2.87	cell proliferation, development
213473_at	BRCA1 associated protein	-2.86	negative regulation of signal transduction
215949_x_at	immunoglobulin heavy constant mu	-2.83	immune response
205668_at	lymphocyte antigen 75	-2.83	endocytosis, immune response, inflammatory response
219688_at	Bardet-Biedl syndrome 7	-2.82	visual perception
218094_s_at	chromosome 20 open reading frame 35	-2.82	protein transport
207845_s_at	anaphase promoting complex subunit 10	-2.8	cell cycle, mitosis, ubiquitin cycle
202265_at	B lymphoma Mo-MLV insertion region (mouse)	-2.79	cell growth and/or maintenance, chromatin modification
208920_at	sorcin	-2.79	heart development, intracellular iron ion storage
218901_at	phospholipid scramblase 4	-2.78	blood coagulation, phospholipid scrambling
208883_at	E3 identified by differential display	-2.75	cell proliferation, ubiquitin cycle
209717_at	ecotropic viral integration site 5	-2.87	cell proliferation, development
218002_s_at	chemokine (C-X-C motif) ligand 14	-2.53	cell-cell signaling, chemotaxis, immune response
208727_s_at	**cell division cycle 42 (GTP binding protein, 25kDa)**	**-2.25**	**protein transport**

IGF signaling pathway

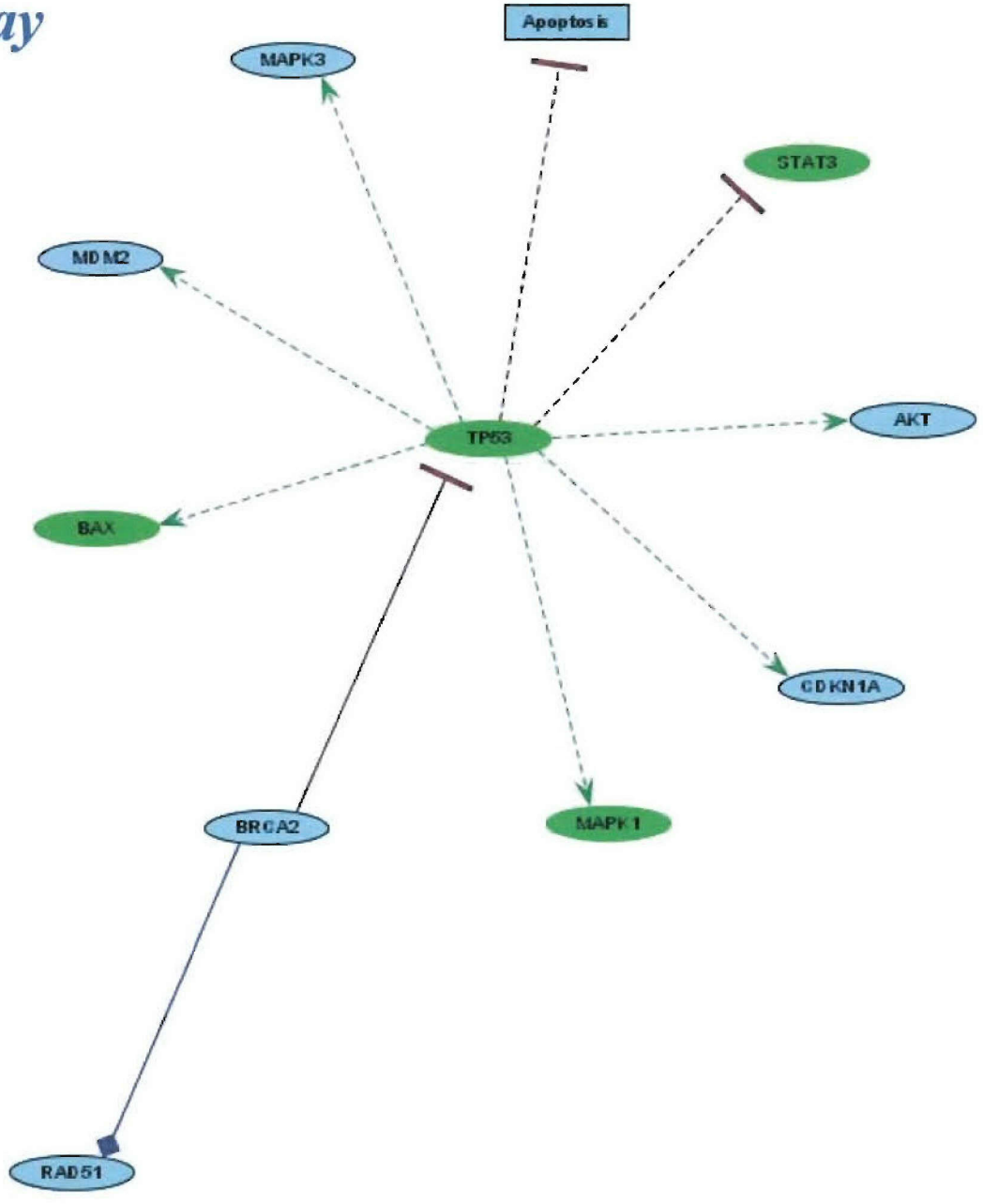


Related genes in this pathway

CDH1 – 20.66
PXN – 5.75
PTPN11 – 5.57 / 4.58
IGF1R – 3.14
MAPK1 – 2.6

Figure 5

p53 signaling pathway

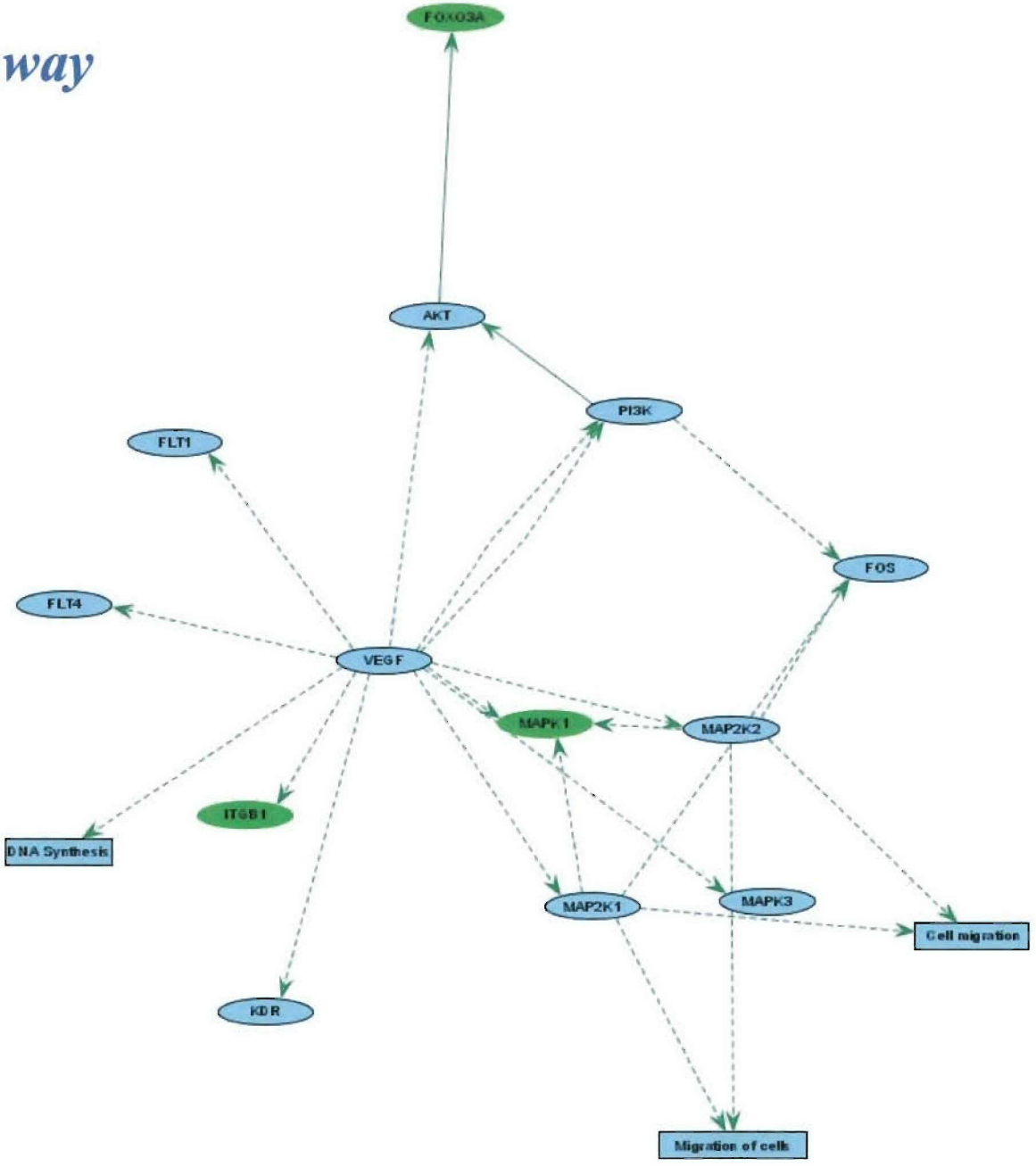


Related genes in this pathway

BAX – 3.65
TP53 – 3.03
STAT3 – 2.62
MAPK1 – 2.6

Figure 6

VEGF signaling pathway

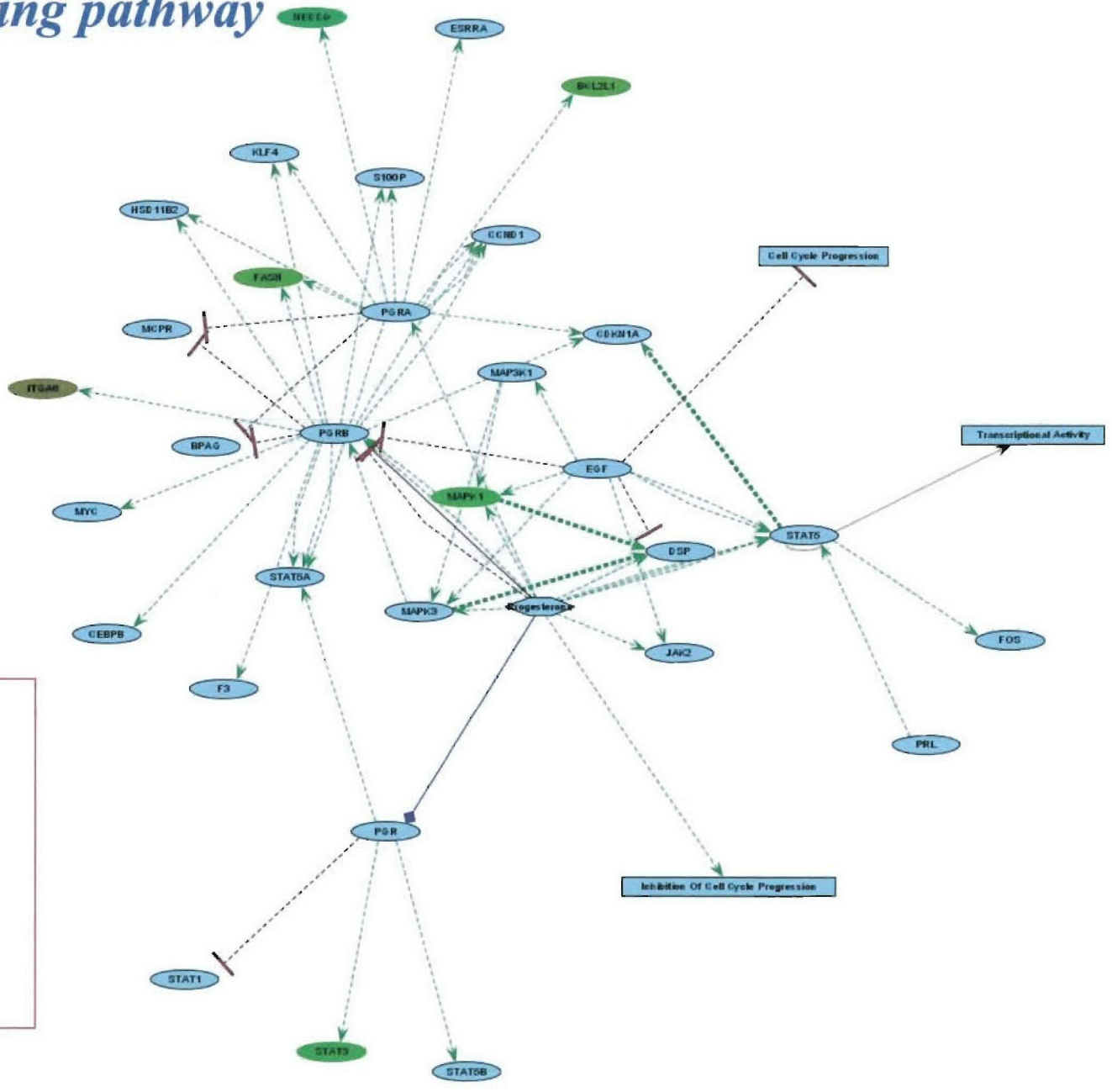


Related genes in this pathway

FOXO3A – 3.33
ITGB1 – 2.76
MAPK1 – 2.6

Figure 7

Progesterone signaling pathway



Related genes in this pathway

Integrin α6 – 10.97
BCL2L1 – 5.4
FASN – 5.15
STAT3 – 2.62
MAPK1 – 2.6
NEDD9 – - 3.39

Figure 8

Verification of the selected RXRα target genes in HMEC

By real-time RT-PCR, and western blot analysis, we confirmed the expression levels of our selected genes in fold induction changes.

Selected genes;

- ***FOXO3A***
- ***E-Cadherin (CDH1)***
- ***BAX***
- ***Paxillin***
- ***STAT3***
- ***Integrin alpha6***
- ***Integrin beta4***
- ***cell division cycle 42 (CDC42)***

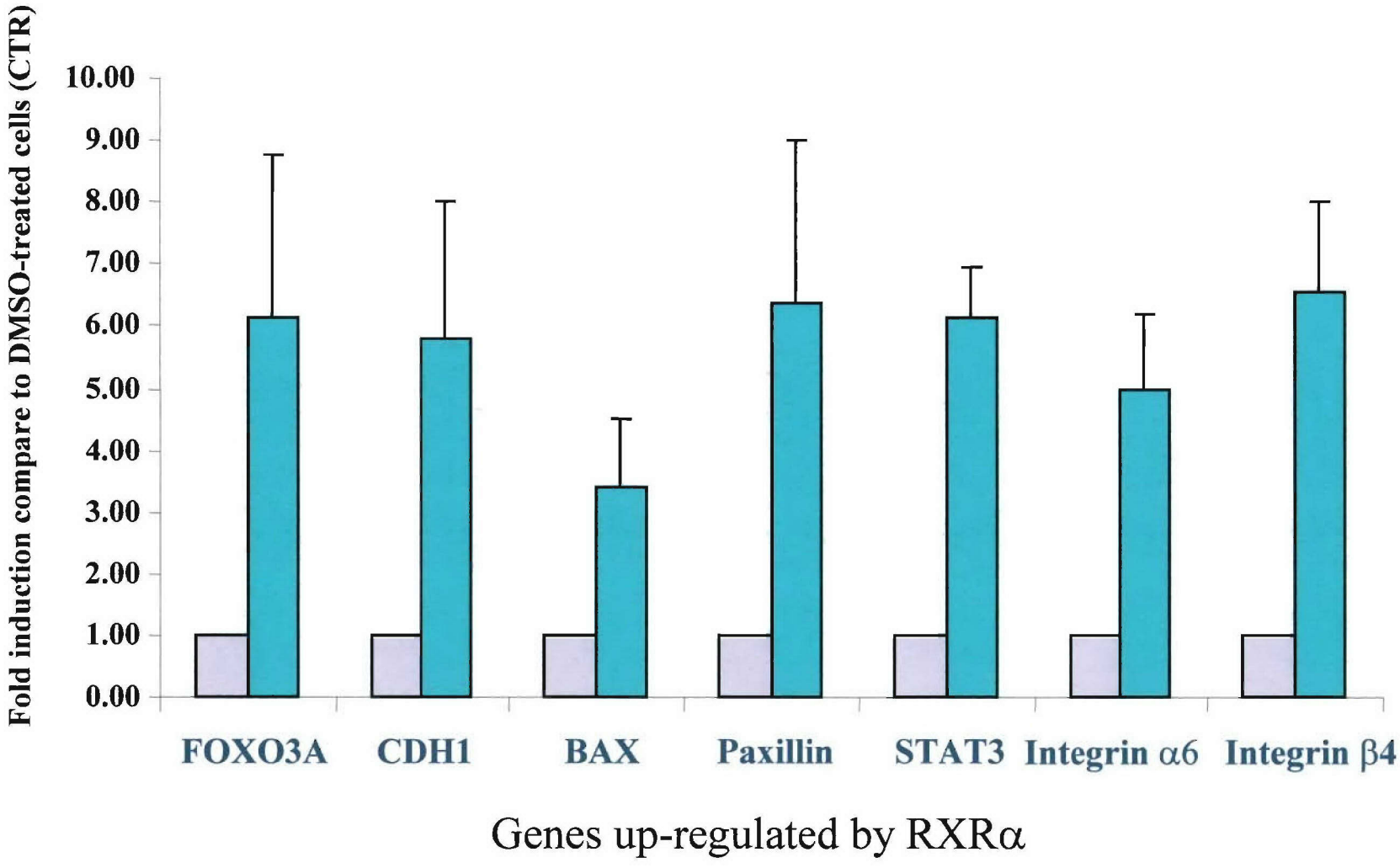

mRNA levels of RXRα-regulated genes in HMEC
(measurement by real-time RT-PCR)
Fold induction compare to DMSO-treated cells (CTR)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
FOXO3A
CDH1
BAX
Paxillin
STAT3
Integrin α6
Integrin β4
Genes up-regulated by RXRα

Figure 9

Protein levels of RXRα-regulated genes in HMEC (measurement by Western blot analysis)

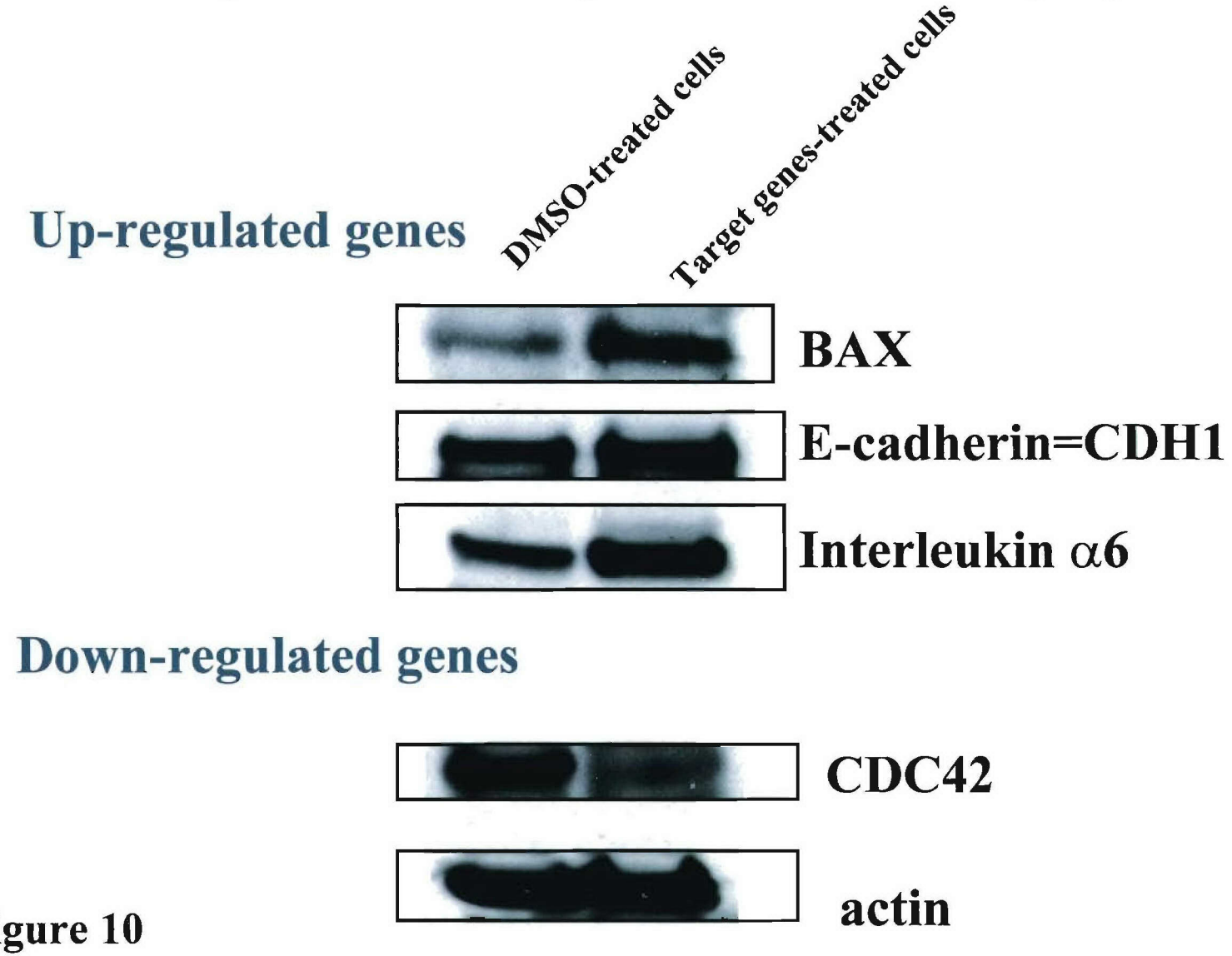


Figure 10

CONCLUSION

1. **The RXR alpha agonist, Ro25-7325 suppressed HMEC cell growth in a dose-dependent manner. Ro25-7325 also inhibited T47D retinoid-sensitive breast cancer cells. Hence, RXR alpha seems to play an important role for growth suppression by rexinoids in breast cells.**

2. **A set of genes regulated by the RXR alpha agonist Ro25-7328 in HMEC were identified. Their expression levels in fold induction were confirmed by using real-time RT-PCR and western blot analysis. Our selected genes may be involved in the suppression of breast cell growth occurred by rexinoids requiring further investigation.**

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Era of Hope meeting